For Reference

NOT TO BE TAKEN FROM THIS ROOM

ABSTRACT

Little is known about the biogenesis or
physiological role of ethylene, the main olefinic volatile
produced by ripening fruit. The research in this thesis
was directed towards a solution of these problems, through
the preparation and study of cell-free systems capable of
ethylene production.

Because of the minuteness of the quantities of
ethylene produced by biological systems, analytical
problems are major ones. To solve these, a highly
sensitive gas chromatographic unit with a hydrogen flame
ionization detector was constructed. A quantitative
procedure for collection and analysis of very low
concentrations of ethylene (1 part in 10^8 parts of air)
was developed.

From gas chromatographic, mass spectrometric,
and chemical evidence, the unequivocal identity of ethylene
as a volatile produced by sub-cellular fractions from
different sources was established.

The preparative procedure for the isolation of
sub-cellular fractions was critically examined and a method
for isolation, from tomato, of particulate fractions active
in ethylene production was outlined. It was shown that
mitochondria are a site of ethylene production. Studies
revealed that structurally "intact" tomato mitochondria
do not produce detectable amounts of ethylene. However,

when the particulate fractions are partially fragmented
by mechanical devices used for tissue homogenization and
suspension, the ethylene-producing system is activated.
Thus, different preparative procedures used by various
laboratories, with varying degrees of production and
retention of fragmented mitochondria, would yield fractions
of varying ethylene-producing activity. This discovery
offers an explanation for the existing controversy over
the ability of particulate fractions from fruits to
produce ethylene. Similar activation of ethylene production
occurred when the particulate preparations were allowed to
age in vitro, or were sonically treated. That ethylene
production was a property of disintegrated particles was
confirmed by studies of the effects of calcium ions,
cholic acid, and phospholipase A, on ethylene production by
"intact" mitochondria. All these reagents activated the
mitochondria with respect to ethylene production, but to
a lesser extent than did sonic treatment.

The particulate fractions of tomato which were
active in ethylene production also were capable of
phosphorylation and carbon dioxide production, although
under the experimental conditions used, no quantitative
relationships among these three properties could be
expressed. Inhibition of ethylene production occurred
when mitochondria, fragmented mitochondria and some
microsomes were isolated together.

Both gas chromatographic and mass spectrometric evidence have been provided to prove that particles from rat liver, rat intestinal mucosa and <u>Penicillium</u> <u>digitatum</u> produce ethylene. By similar techniques, the presence of ethylene in the exhaled gases of normal human subjects was also shown. These findings are the first report of ethylene production by particles other than those of fruits. A view has been expressed that ethylene production may be a phenomenon common to all living things, and not restricted to fruits and microorganisms, as hitherto believed.

With knowledge of the site of ethylene production, and a technique for activation of the system involved, progress in solving the problems of the modes of production and action of this gas in biological systems should now be more rapid.

THE UNIVERSITY OF ALBERTA

STUDIES ON ETHYLENE PRODUCTION

BY SUB-CELLULAR FRACTIONS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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INTRODUCTION

Since the discovery of ethylene as the main
olefinic constituent of fruit emanations (1,2), many
painstaking studies on the identification of ethylene
and its physiological role in fruit ripening and
respiration have been made. The many analytical
methods developed for the estimation of ethylene are
reviewed in a later section. Porritt (3), Biale (4,5),
Pentzer and Heinze (6), Ulrich (7) and recently Pratt
(8) and Varner (9) have written comprehensive reviews
on the metabolism of ethylene in fruits with particular
stress on its physiological role. Hence, these aspects
will be dealt with only briefly here, and in the
present review emphasis will be placed on ethylene
production in cell-free systems.

As most fruits ripen, they respire rapidly
and give off ethylene. The sudden rise in respiratory
rate associated with ripening is called the climacteric,
and is indicative of a transition stage in the
development of the fruit, between ontogeny and senescence.
Among the volatile emanations (4,6), ethylene has been
identified as the main olefinic substance (10,11,12).
Fidler (13) has calculated that, on the basis of carbon,
70-80% of the total volatile (other than carbon dioxide)
is ethylene. The production of ethylene by leaves and

flowers (4) and also by certain microorganisms such as
Penicillium digitatum has also been reported (14,15,16).

A hormonal role for ethylene has often been
advanced (5,7,17,18,19). It has been suggested as
playing a part in many important physiological processes,
such as the acceleration of abcission, shedding of
'buttons' (calyx and receptacle), increase of cell
permeability, promotion of epinastic effects and of
stem end decay, and reduction of storage life. It has
often been implicated as the causative agent ("vivo toxin")
for apple scald and wilt diseases (20). Small amounts of
ethylene stimulate respiration and induce ripening
(4,5,6,7).

The role of ethylene in fruit ripening is
controversial. One theory is that ripening occurs when
the concentration of ethylene in the fruit become
sufficiently high, that is, that ethylene causes ripening.
Administration of minute amounts (0.2 to 1.0 ppm) of
ethylene to unripe fruits will accelerate ripening (4).
Although it hastens the onset of the climacteric,
ethylene does not affect the progress of this change
once it is initiated (4,5). The alternative theory (21)
is that ethylene is merely a by-product of the ripening
process, with no causal role in normal ripening. Buhler,
Hansen and Wang (21) from a study of the uptake of
labelled ethylene of high specific activity suggested

that ethylene is probably the terminal product of a
chain of metabolic processes in fruits and cannot be
further metabolised.

In efforts to clarify the metabolic role of
ethylene, several investigators have studied the
relationships among ethylene evolution, respiration,
and ripening. The following are some of the more
significant relationships discovered. In ripening
tomatoes (22), as well as in many other fruits (10,17,23),
a climacteric peak in carbon dioxide production
accompanies the ethylene evolution. Both ethylene
production and ripening are aerobic processes (4,5,22).
Ripening tomatoes under an atmosphere of oxygen produce
nearly double the amount of ethylene that they do in
air (22). Furthermore, the onset of climacteric under
oxygen is nearly ten days ahead$_\wedge$ of normal. These findings lend
support to the view that ethylene production is closely
related to respiratory processes. Its production
in vivo is held to be autocatalytic (4) and its
physiological role in ripening that of shifting the
events of senescence on the time axis (4,5,7,9).

The role of phosphorylation in ethylene
production and ripening was investigated by Spencer (24).
Infiltration of 2,4-dinitrophenol (DNP) an uncoupling
agent into whole tomato fruit resulted in a large
increase in carbon dioxide evolution, depressed ethylene

evolution, and resulted in failure to ripen. Marks,
Bernlohr, and Varner (25) using P^{32} showed that maximum
oxidative phosphorylation, which is reached in the
preclimacteric tomato fruit, is maintained during the
postclimacteric period of senescence. DNP treatment
affected the normal ripening of fruits. Such results
(5,24,25,26) suggest a requirement for oxidative
phosphorylation for ethylene production and fruit
ripening. Although considerable evidence has accumulated
for a close relationship between climacteric respiration
and oxidative phosphorylation (5,9), the fundamental
relationship between respiratory processes and ethylene
production remains to be elucidated.

Apart from gross changes in ethylene production
during the ripening process, little is known about the
biosynthesis of the gas or about the mechanism by which
artificially supplied ethylene hastens the onset of
the climacteric in many fruits. Recent attempts to solve
these problems have centered on studies of ethylene
production by tissue slices and cell-free systems.

Hall (27) absorbed the volatile emanations of
crude extracts of apple and Penicillium digitatum directly
in potassium permanganate solution. He obtained high
amounts of volatiles, which he presumed to be ethylene,
when substrates like arabinose and pectins were added to
the extracts. He postulated simple sugars and organic

acids as possible precursors of ethylene. While this
work was criticized on many accounts, such as the lack
of specificity of the analytical method and the
possibility of contamination, Stolwijk and Burg (28)
confirmed ethylene production in crude extracts of
apple and Penicillium digitatum. They used a gas
chromatographic technique with a katharometer detector
and suggested that ethylene arose from many substrates,
and especially from alcohols. Fergus (14) implicated
mannose, mannitol, and citric acid, while Phan Chan Ton
(15) added glucose, glycerol, and alanine to the long
list of possible precursors of ethylene in cultures of
Penicillium digitatum. In recent years, Burg (29,30),
Burg and Thimann (26,31,32) and Burg and Stolwijk (33)
have investigated ethylene production from slices and
plugs of apple fruit. From the use of a variety of
experimental conditions, metabolic inhibitors, and
labelled metabolites, they concluded that the ethylene-
producing system was located in a particulate fraction
of the cells. Furthermore, they inferred ethylene
production was connected to the respiratory process
through a terminal oxidase and was dependent on
oxidative phosphorylation. Burg (30) obtained a
relatively high ratio of specific activity of ethylene
to that of carbon dioxide when the apple tissue was
supplied with uniformly labelled sucrose. However, his

main conclusions (30,32) were that none of the intermediates of the Embden-Meyerhof pathway, Krebs' tricarboxylic acid cycle, pentose phosphate pathway or pathway of formation of shikimic acid is a direct precursor of ethylene. From studies of the conversion of glucose labelled either uniformly or specifically, Burg and Thimann (32) did find the C-6 of glucose three times more effective than the other five carbons of glucose in labelling ethylene. Earlier, Burg and Thimann (31) had postulated that the formation of one ethylene precursor is favoured by anaerobic conditions, whereas another unknown intermediate requires aerobic conditions. The final step in the synthesis of ethylene is thought to involve a reversible dehydration of this unknown intermediate compound. Recently, Varner (9) has suggested acrylic acid as a possible precursor of ethylene.

An interesting approach to the problem of ethylene metabolism in fruits was made by Buhler et al (21). They exposed several kinds of fruits to 1 ppm atmosphere of labelled ethylene of high specific activity. After many days, low but measurable incorporation of radioactivity was obtained in the organic acid fractions of only pears and avocadoes. According to them, even this low uptake was due to exchange reactions, and ethylene once formed is not capable of further metabolism.

Earlier work suffered from lack of a sensitive, specific method for ethylene assay and also from the possibility of contamination of the tissue with ethylene-producing microorganisms (34). Apart from work on whole fruit, tissue slices, and crude extracts, in the last three years, considerable interest has been shown in the production of ethylene by sub-cellular particles. Such investigations became possible due to the introduction of highly sensitive gas chromatographic and other techniques for ethylene analysis.

Burg and Thimann (26,31) from studies on apple plugs suggested cytoplasmic particles as the active site and Spencer (35) showed that mitochondrial preparations from tomatoes in the advanced turning stage of ripening were active in ethylene production. However, a controversy has now developed regarding attempts by several laboratories to prepare cell-free systems capable of ethylene evolution. Meigh, Norris, Craft, and Lieberman (36), under conditions very different from those employed by Spencer (35), were not able to obtain production of ethylene from sub-cellular fractions of either tomatoes or apples. The amounts of ethylene recorded from mitochondria or microsomes were negligible as compared to the amounts obtained from whole homogenates. Lieberman and Craft (37) isolated particles from apples and tomatoes under acidic conditions (38) and were able

to detect extremely small amounts of ethylene when
thiomalic or thioglycolic acid was added to the reaction
mixture. Burg and Burg (39) using gas chromatographic
evidence, have claimed that the volatile substance
produced by the "acidic" particles of Lieberman and
Craft was not, in fact, ethylene. In view of the above,
it would be appropriate to repeat Smock's (40) statement
that "Probably today no aspect of post harvest physiology
of fruits is as controversial as that of the role of
ethylene, and its biogenesis in vitro".

While the production of ethylene by fruits
and certain microorganisms has been the subject of
extensive investigation, the possible role of this compound
in the metabolism of animals has been virtually ignored.
Early in our studies we attempted the collection of
ethylene from rat exhalations, but found that practical
difficulties in the maintenance of truly aseptic conditions
in the respiration chamber, etc., made it impossible to
draw any valid conclusions. Very recently Kakanov (77),
using a bio-assay technique and mass spectrometric
analysis, detected ethylene in air passed through chambers
containing rats or swine ascarids. However, no mention
was made of purification of the air prior to passage
through the chambers, or of control experiments. His
results did indicate, nevertheless that sub-cutaneous
transplantation of tumours resulted in a 4-5 fold increase
in ethylene production as compared to healthy animals.

With the discovery of mitochondria as an active site of ethylene production (35), further investigations were undertaken in this laboratory to develop a sensitive analytical method for ethylene and to study the biogenesis of this gas in cell-free systems. One part of the present work involved the development of a highly sensitive and quantitative gas chromatographic technique which made possible the study of ethylene production in cell-free systems. The main theme of this investigation is a critical study of the production of ethylene by, and some of the biochemical properties of, the different sub-cellular particles of tomato. The scope of the study has been extended to ethylene production by the particles from rat liver, rat intestinal mucosa, and Penicillium digitatum, and the ethylene content of the respiratory gases of human subjects.

MATERIALS AND METHODS

MATERIALS

Tomatoes

Apple and tomato fruits have been the favourite choice for study (35,36,37,39) of ethylene production in cell-free systems. In the present investigation, tomato fruit (Variety"V121") in the advanced turning stage (half to three-quarters of the surface red) and firm ripe stage (more than three-quarters red) was selected, since ethylene evolution has been shown to be at a maximum at these stages (22). The fruits were grown at the University of Alberta greenhouses, graded immediately after harvest according to ripeness (22), and stored in the frozen state. Periodic comparison experiments were made with fresh fruits when these were available. In some experiments, we had to use a commercial variety (Mexican) of tomatoes, and these have been noted in the text.

In all the experiments, from 850-900 gm. of randomly sampled fruit was used as the starting material to prepare the initial homogenate for isolation of sub-cellular particles. Trial experiments showed that both fresh and frozen fruits could be used with equal success to prepare particulate fractions capable of ethylene production. Hence, frozen fruit was used in all the experiments, reported in this investigation.

Rat Liver and Intestinal Mucosa

About 50 gm. fresh weight of liver and 2.5 gm. intestinal mucosa, pooled from four rats, were used in each experimental run. Adult female "Wistar" rats, 10 weeks old were killed by decapitation immediately prior to the experiments. As recommended by Dr. J. Tuba, Department of Biochemistry, a 10 cm. loop of the small intestine posterior from the pyloric sphincter was dissected and washed with demineralised water and blotted dry. The mucosa, obtained by squeezing with a spatula, was used as the intestinal mucosa sample.

Penicillium digitatum (Sacc) NRRL 1203

The fungus was grown in stationary cultures, at 37°C. under aseptic conditions. The growth medium (41) consisted of glucose (25.7 gm.), ammonium nitrate (4 gm.), potassium dihydrogen phosphate (13.61 gm.), magnesium sulphate (1.23 gm.), ferric nitrate (5 mg.) and 1 ml. of Pratt's micro-element solution (42), dissolved in 1 liter of distilled water. The pH of the medium was 4.4. Two-week old growth (approx. 25 gm.) was harvested and either used immediately or frozen. The pH of the medium at harvest was 4.2.

Respiratory Gases of Human Subjects

Expired air samples from adult male and female subjects, fasted 10 hours and unfasted, were collected in a 100 liter Douglas bag, and then pumped through mercuric

perchlorate solution in the usual manner (described later)
for absorption of ethylene. Control samples were run with
room air pumped into the Douglas bag.

METHODS

Preparation of Sub-cellular Fractions of Tomatoes

The preparative procedure for the isolation of
the different sub-cellular fractions was based on the
method used previously in this laboratory for mitochondria (35).

All operations in the preparative procedure were
performed at 0°C. under aseptic conditions.

The fruit was first ground with an equal weight
of phosphate buffer (0.5M potassium dihydrogen phosphate,
0.25M sucrose, adjusted to pH 8.0 with sodium hydroxide)
at a low speed in the "Waring" blendor, and the homogenate
filtered through four folds of cheese cloth. This buffer
rapidly neutralised the high acidity of the fruit and
brought the pH of the filtrate to 7.0. In our more recent
work, we have used 0.5M sucrose (instead of 0.25M) in the
initial phosphate buffer, for reasons which will be
discussed later. The various cellular fractions were
isolated from this filtrate by differential centrifugation
in the "Spinco" Model L Ultracentrifuge, according the
scheme given in Figure I.

Cell fragments, chloroplasts, and nuclei were
obtained by centrifugation of the homogenate at 4,000g for 7
minutes. As a routine procedure "mitochondria" were
sedimented at 35,000g for 15 minutes, for reasons discussed
later. Another fraction separated at 40,210g for 15 minutes
undoubtedly consisted of a mixture of fragmented mitochondria

Figure I

SCHEME FOR THE PREPARATION OF SUB-CELLULAR FRACTIONS

Tomato fruit

    ground with phosphate buffer (0.5$\underline{M}$ KH_2PO_4, 0.25 or 0.5M sucrose, pH 8.0) and filtered through cheese cloth.

Residue   Filtrate

(rejected)

    centrifuged at 4,000g for 7 minutes.

Cell fragments-   Supernatant

nuclei, chloroplasts

    centrifuged at 35,000g for 15 minutes.

Mitochondria   Supernatant

    centrifuged at 40,210g for 15 minutes

"Fragmented mitochondria and microsomes"   Supernatant

    centrifuged at 105,000g for 10 minutes

"Microsomes"   Supernatant

(soluble fraction)

and microsomes (fragments of endoplasmic reticulum). In
experiments where a "combined mitochondrial-microsomal"
fraction was used, it was obtained by direct centrifugation
at 40,210g for 45 minutes of the supernatant from the
separation performed at 4,000g. The remainder of the
"microsomes" was sedimented at 105,000g for 10 minutes.
The soluble fraction was the supernatant liquid remaining
after this centrifugation.

All the solid fractions designated as "washed"
were suspended in 90 mls. of phosphate buffer (0.01M potassium
dihydrogen phosphate-0.5M sucrose, adjusted to pH 7.0 with
sodium hydroxide) with the help of a Servall "Omnimixer"
run at a low speed. These particulate fractions were
washed only once and then recovered at the appropriate
centrifugal force.

The different cellular fractions were suspended
in 25 ml. of the buffer substrate mixture of the following
composition: 0.5M sucrose, 0.125M potassium dihydrogen
phosphate, 10^{-3}M magnesium sulphate, 10^{-3}M manganese sulphate,
0.25M l-malic acid (0.05M in our more recent work), pH 7.0
(adjusted with sodium hydroxide). The reaction mixture was
sterilized prior to use. 1.98×10^{-3}M adenosine triphosphate
(ATP) and 3.3×10^{-4} M diphosphopyridine nucleotide (DPN),
both supplied by Sigma Chemical Company, were added after
sterilization.

As a routine procedure, 20 ml. of the particle: buffer: substrate suspension was used for ethylene collection; the remainder was used for other determinations.

Preparation of Particles from Rat Liver and Intestinal Mucosa

The tissues were homogenized in ice-cold 0.88M sucrose (10 gm. wet tissue/100 ml. suspension) at a low speed in the Servall "Omnimixer". The pH was maintained at 7.0-7.2 by addition of 0.1N KOH when necessary. The sediment from centrifugation at 4,000g for 7 minutes was discarded. The particles tested for ethylene-producing activity were separated by centrifugation at 12,728g for 15 minutes, and suspended in 25 ml. of the same reaction mixture which was used for tomato mitochondria (page 15).

Preparation of Particles from Penicillium digitatum (Sacc)

Mycelia were homogenized in ice-cold 0.5M sucrose: 0.1M potassium dihydrogen phosphate buffer, pH 7.0 at a low speed in the Servall "Omnimixer". The homogenate was centrifuged at 4,000g for 7 minutes. The sediment was rejected and the supernatant was recentrifuged at 12,725g for 15 minutes. The sediment was suspended in 25 ml. of the same reaction mixture which was used for tomato mitochondria (page 15).

Treatments of Sub-Cellular Fractions

For preparation of "intact" and "sonically treated" fractions, the particulate fractions were suspended in the reaction mixture with the help of a heavy

glass rod followed by a magnetic stirrer operated at low speeds. Particles to be sonically treated were then subjected, at 2°C., to four minutes in a 250 W, 10 Kc. Raytheon sonic oscillator tuned to 1.1 amperes. "Partially disintegrated fractions were prepared by suspension of the particulate fractions in ice-cold buffer-substrate mixture by the use of a Servall "Omnimixer" run at a very low speed.

When particles were treated with phospholipase A and/or Ca^{++}, they were suspended in the reaction mixture (page 15) containing Ca^{++} at a concentration of 3 mM, with the help of a magnetic mixer operated at low speeds. Lyophilized snake venom (Agkistrodon p. piscivorus, Ross Allen's Reptile Institute, Florida) was used as a source of enzyme. This preparation was obtained from Dr. H.B. Collier, Department of Biochemistry, who had found it to be active in the conversion of lecithin to lysolecithin. Prior to use, the venom (25 mg.) in 5 ml. of the buffer-substrate mixture (page 15) containing 3 mM Ca^{++} was boiled for 15 minutes to inactivate enzymes other than the phospholipase A (43,44,45). The presence of Ca^{++} is necessary for activity of phospholipase A (45). The final concentrations of particles and chemicals in the substrate were kept the same as in untreated preparations. The venom was present in the reaction mixture in amounts of approximately 1 mg./ml. Hereafter the heated snake venom preparation will be referred to as phospholipase A.

Collection of Ethylene

A major difficulty in the study of ethylene production by cell-free systems is the extremely low rate of its emanation. Consequently, the analytical problems involve the concentration and purification of the volatiles before a satisfactory analysis is possible.

In the present investigation, ethylene produced by the sub-cellular fractions was collected by the method described by Spencer (46). Certain modifications were included in the micro-collection apparatus to increase its efficiency at very low concentrations of ethylene.

The reaction vessel was a 100 ml. extraction flask fitted with glass tubes for air inlet and outlet. Air supply from a compressed air tank was metered and purified by passing through columns (12 x 1 inch) of mercuric perchlorate solution, glass wool, brominated activated carbon (47,48) activated carbon, glass wool soaked in glycerol, and dry cotton wool, all connected in series. Such a supply of air purified from unsaturated hydrocarbons and possible contaminations from microorganisms, was passed into the reaction vessel at the rate of 5 ml. per minute. The outlet end of the reaction vessel was connected to a medium porosity micro-filter stick (Microchemical Specialties Co., Cat. No. 7310) of 2 mm. tip diameter. The filter stick reached to the bottom of an absorption tube which contained 4.5 ml. of 0.25M mercuric perchlorate in

2.OM perchloric acid (34) and 5 microliter of n-butanol.
The absorption tube (Figure II) was 47 cm. long, 9 mm.
inside diameter, and had a 3.2 cm. diameter bulb 27 cm.
from the bottom. The bottom of the absorption tube was
fitted with a medium porosity alundum filter disc (Fisher
Scientific Co., Cat. No. 9776) of 2 mm. tip diameter.
The alundum disc was connected to a separate air supply
(purified as described above) at the rate of 20 ml. per
minute.

The reaction vessel was maintained at 25°C.,
while the absorption section of the micro-collection
apparatus was packed in ice in a thermos bottle.

The contents of the reaction vessel were
constantly and slowly stirred either with a "gas-tight"
stainless steel stirrer (Fisher Scientific Co., Cat. No.
14-512) or a magnetic stirrer, connected to a powerstat.
To assist in the maintainance of a steady flow rate over
extended periods of time, a four inch long thermometer
capillary tube was connected between the gas regulating
valve and the flow meter.

Analysis for Ethylene

Ethylene collected in the mercuric perchlorate
solution as a mercury complex was determined by three
different procedures: micromanometric, mass spectrometric,
and gas chromatographic.

AIR INLET FROM REACTION VESSEL

2 mm

AUXILIARY AIR SUPPLY INLET

9 mm

3.5 cm

47 cm

27 cm

GLASS MICROFILTER STICK
(2 mm TIP DIAMETER)

ALUNDUM DISC (2 mm TIP DIAMETER)
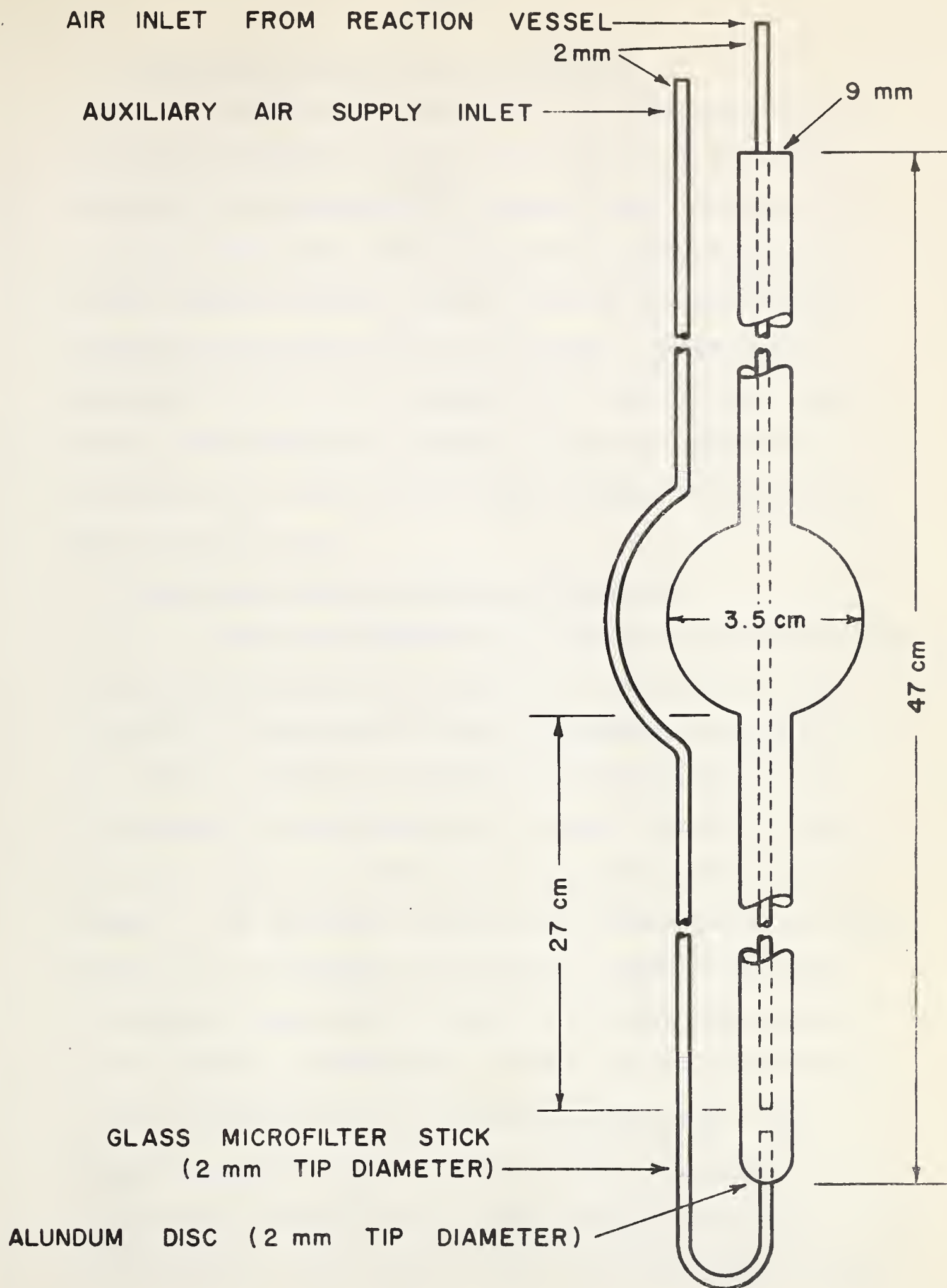
FIG. II.

ABSORPTION TUBE FOR COLLECTION OF ETHYLENE.

1. Manometric Determination of Ethylene

Ethylene was quantitatively determined by the manometric procedure devised by Young et al (34) and adapted to the microscale by Spencer (46). The gas was released from 4 ml. of the ethylene mercury complex inside special warburg vessels (49) by the addition of 4M LiCl in the ratio of 1:4 by volume. Differential manometers were used to measure the volume of the gas at 25°C. and atmospheric pressure. The micromanometric procedure was quantitatively reproducible for amounts of gas as low as 8 μl.

2. Mass Spectrometric Analysis of Ethylene

The gas, liberated from the mercuric perchlorate absorbing solution by the addition of 4M LiCl (1:4 by volume) was subjected to mass spectrometric analysis. In order to achieve a satisfactory performance of the instrument, it was essential to remove traces of carbon dioxide, water, and other polar substances from the gas sample. The liberated ethylene was passed through columns (12" x 1") of drierite and ascarite, followed by a dry ice-acetone trap, and by means of nitrogen carrier gas (10 ml./minute) condensation of the purified ethylene in a trap cooled by liquid nitrogen was achieved. The liquid nitrogen cooled collection trap consisted of coiled glass tubing 136 cm. long and 6 mm. diameter, fitted with a 12/30 joint at both inlet and outlet ends.

The outlet end of the liquid nitrogen cooled trap was connected to a battery of columns similar to those at the inlet end. This prevented air and other impurities from getting into the ethylene trap by back suction.

In our later work, we modified the above method of ethylene collection to a simpler and more effective procedure outlined as follows: Ethylene was liberated inside serum bottles and representative samples were injected into an evacuated tube (6 x 0.5 cm.) fitted with a 12/30 joint on one end and serum stopper on the other end. The tube was cooled in liquid nitrogen and evacuated to 6 mμ mercury prior to injection of the ethylene. After liquefaction of the sample, the tube was transferred to a dry ice-acetone bath. After a few minutes to allow warming to dry ice temperature, the samples were introduced into the mass spectrometer for analysis. The method of introducing samples into the mass spectrometer involved fitting the 12/30 joint end of the ethylene trap (in dry ice) to the spectrometer and drawing samples into the previously evacuated spectrometer. Mass spectrometric analysis was done in the Department of Chemistry under the supervision of Dr. P. Kebarle.

Many recovery and control (aerated mercuric perchlorate solution) experiments showed only trace amounts of carbon dioxide, water, and complete absence of hydrocarbons other than ethylene. No ethylene was detected in the controls.

The limit of ethylene detection by our earlier procedure was $1:10^6$, while with the simplified procedure, the limit of detection for ethylene was $1:10^8$.

Results of the analyses were interpreted by comparison with the cracking pattern of authentic samples of ethylene and suitable control samples. In the present investigation, mass spectrometric analysis has been used to provide corroborating evidence that the gas liberated from mercuric perchlorate solution was ethylene. The mass spectrometric analysis also proved useful in eliminating the possibility of the presence of other olefines, either from the reaction products or as contaminants.

3. Gas Chromatographic Analysis of Ethylene

A highly sensitive gas chromatographic unit with a hydrogen flame ionization detector was built. The construction of the gas chromatographic unit and the method finally adopted for quantitative analysis are described below. The limit of detection by this method is 1 part of ethylene in 10^8 parts of air or gas sample. Results relating to development and testing of the unit are discussed on page 36.

A. Construction of Gas Chromatographic Unit

The entire gas chromatographic unit consisted of the following components: analytical and reference columns, a pair of hydrogen flame ionization detectors, an amplifier, and a recorder.

Columns

The analytical column (50) was a coiled metal tube 406 cm. long and 5 mm. in diameter. The column packing consisted of 30-60 mesh Johns-Manville C_{22} firebrick impregnated with 30% of its weight of squalane as the stationary phase. The reference column was U-shaped, and 61 cm. long and 2 mm. in diameter. It was packed with firebrick of sufficiently fine mesh to offer the same resistance to gas flow as the analytical column. The columns were heated at 100°C. for several hours and flushed continuously with nitrogen gas for several days. This reduced the volatility of the stationary phase, and thus the background, to a minimum. It was not found necessary to flush the analytical column with nitrogen at all times when the column was not in use, but only for a few hours immediately prior to analysis. It was kept sealed from entrance of air at all times. Each column was connected to a hydrogen flame ionization detector on one end and to a common source of carrier gas mixture at the other end. The sample inlet on the analytical column was closed with a self sealing stopper. The columns were kept at room temperature. The columns had the desirable characteristics of long life and minimum volatility of the stationary phase.

Detectors

The hydrogen flame ionization detector (Figure III) constructed by us was based on the general design of McWilliam and Dewar (51) as described by Meigh (50).
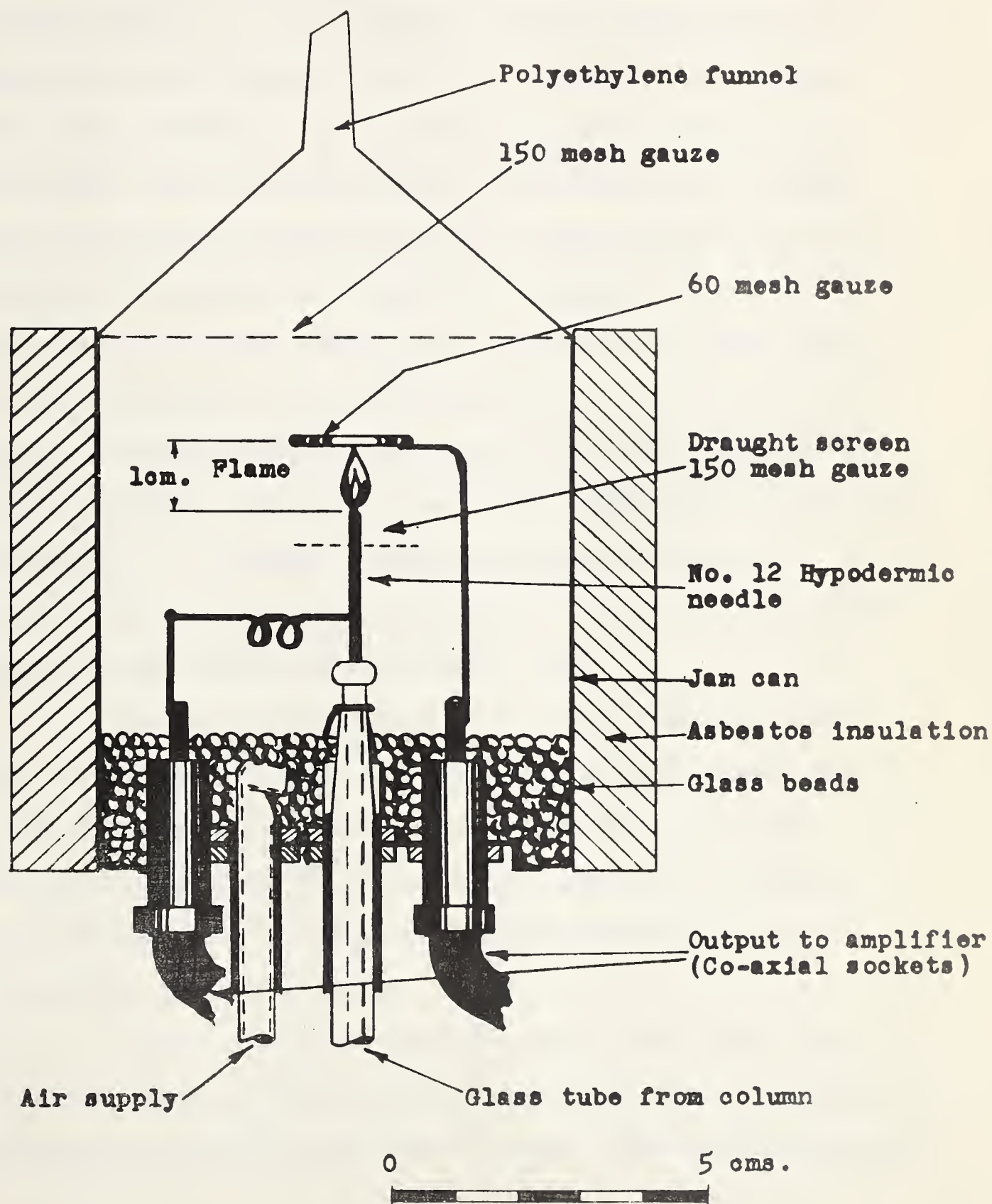
FIGURE III.

HYDROGEN FLAME IONIZATION DETECTOR

The main structural unit of the detector was a jam can fitted with a funnel. The issuing carrier gas was burned at a jet made from a 12 gauge hypodermic needle cut square at the tip. The needle also served as the positive terminal. The negative terminal consisted of a piece of 60 mesh circular (1.5 cm. diameter) brass gauze placed approximately 1 cm. above the needle. A piece of fine copper mesh placed below the flame acted as a draught screen and prevented deterioration of the insulators due to deposition of combustion products. Current from a 300 V source was fed to the terminals. A draft-free supply of air (40 ml./minute) for combustion and prevention of moisture condensations was maintained from the base of the detector through a circular perforated tube having glass beads piled over it. A steady dust-free supply of air was obtained from air tanks by introducing a very fine porosity sintered glass funnel in the supply line.

The lid of the detector was made completely air tight, since dust or smoke entering the chamber contribute greatly to background noise. The background noise was completely eliminated by fully insulating the structural unit with asbestos and sealing all metal-to-glass joints with plastic aluminum cement.

In the gas chromatographic unit, we used a pair of hydrogen flame ionization detectors, one for the analytical column and one for the reference column. The main advantages

in employing a pair of hydrogen flame ionization detectors
rather than a single one were simplification of the design
of the amplifier and cancellation of signals due to any
change in the composition of the carrier gases. (By
removal of these signals operational stability was improved).

Amplifier

In the ionization detectors, the hydrogen flames
are maintained across a potential of 300 V. The flames
offer a high impedence circuit. The amplifier, is therefore,
a high voltage gain amplifier with a negative phase shift
(52). As shown in Figure IV, one flame (the reference side)
feeds one grid, while the analytical flame feeds the other
grid. The circuit is completed by having the negative ends
of the detectors connected to the twin triode (Philips,
MiniWatt 6085).

In order to screen the amplifier from stray
magnetic fields, it was housed in a metal box. Co-axial
cables were used to connect the amplifier with the detector
on one end and the recorder on the other end. Socket
arrangements were made on the amplifier to feed known
electrical signals to the grid, for purposes of calibration.
Known signals were obtained from standard mercury cells
(1.3 V, DC), suitably attenuated.

Recorder

We employed a Sargent MR recorder, which had a
built-in range switch. By proper selection of the range

FIGURE IV

AMPLIFIER FOR FLAME
IONIZATION DETECTOR
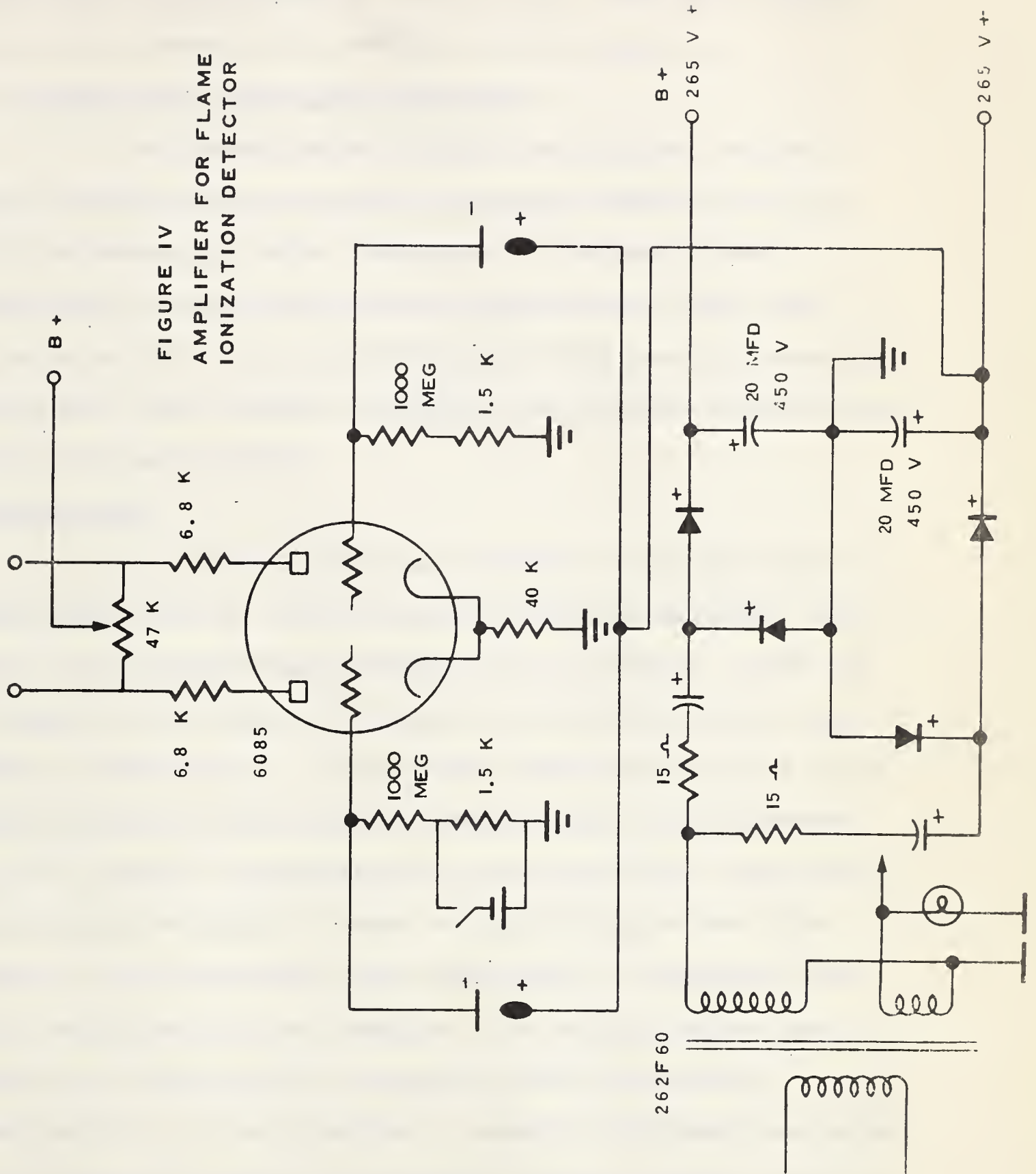
switch, it was possible to suitably attenuate the input
signals from the amplifier. Further, operational
stability was achieved by introducing a 1000 ohms resistor,
in series, between the recorder and the amplifer.

B. Quantitative Analysis of Ethylene

The quantitative analysis of ethylene by the
gas chromatographic procedure involves: operation of the
gas chromatograph under conditions of maximum column
efficiency compatible with the overall sensitivity and
electrical stability; calibrations of the gas chromatographic
apparatus with suitable standards; and finally interpretation
of analytical results.

Carrier Gas

Our carrier gas was a mixture of nitrogen and
hydrogen, each at a flow rate of 15 ml./2.6 seconds. The
gases were individually metered prior to mixing. Both the
analytical and reference columns received the carrier gas
from a common source. The column length and gas flow rates
were adjusted to make them compatible with the efficiency
of the column, the sensitivity of the detector, and overall
electrical stability. Under the conditions of our flow
rates, the column had a high efficiency of separation and
low retention time for ethylene. At concentrations less
than 1 μl. ethylene/ml., spreading and tailing were
negligible. It was possible to separate ethylene from other
low molecular weight hydrocarbons, carbon dioxide, and air.

Ethylene Standards

Ethylene standards for gas chromatography were prepared from manometrically standarised stock solutions of ethylene:mercuric perchlorate (34). These stock solutions were obtained from 1:500-1:1000 dilutions of a saturated solution of ethylene:mercuric perchlorate. The saturated solutions were prepared by dispersing pure ethylene gas (99.9%, Ohio Manufacturing Co.) in 25 mls. of cold mercuric perchlorate solution containing trace amounts of n-butanol as foaming agent. Standards for gas chromatography were prepared by serial dilutions of standard stock solutions assaying from 10 to 20 μl. ethylene/ml. Mercuric perchlorate solution (34) was used for making dilutions. Standards prepared in the gas phase by serial dilution of pure ethylene gas were found to check well with the above standard solutions.

The ethylene:mercuric complex has a very small dissociation constant at 5°C. (34). In dilute solutions, the complex was found to be stable for one week, at 5°C.

Method for Liberation and Injection of Gas Samples

As in the micromanometric procedure, the gas was quantitatively regenerated from the complex by the addition of one-quarter volume of 4M lithium chloride. The gas was liberated inside capped serum bottles. From the calibration of the serum bottle, ethylene concentration in the gas phase was calculated. Representative 1 ml. gas samples were drawn into a gas-tight syringe (Hamilton Co. Inc., Cat. No. 1001) fitted with a hypodermic needle 25 G 5/8", and injected into the analytical column.

Calibration of the Gas Chromatograph

For quantitative analysis of ethylene in the gas samples, the gas chromatographic unit was calibrated as follows:

Known electrical signals (1-5 mV) were fed at the flame end of the amplifier to calibrate the recorder scale. Signals were obtained with standard mercury cells (1.3 V, DC) suitably attenuated. As a routine procedure, the recorder was attenuated to 1.25 to 2.5 mV full scale deflection, and ethylene concentration (0.01 to 1.0 μl/ml. of gas sample) was related to peak height. Figure V, Curve 3, is an illustration of a calibration curve. Between the above concentration ranges of ethylene the response was always linear and reproducible.

Interpretation of Gas Chromatographic Analysis

The first 1 ml. gas sample obtained from the serum bottle was used to calculate the total ethylene concentration. The identity of the sample was established on the basis of retention time and co-chromatography with authentic ethylene samples. In some analyses, disappearance of the ethylene peak on passage through activated and brominated carbon columns was used as added confirmation of the identity of the gas.

The retention time was recorded as the interval between sample injection and response of the recorder pen. Standards and unknown samples were always analysed together,

within a week of their collection. Ethylene, once
regenerated inside a capped serum bottle, was found to
keep well in the gas phase for 1-2 hours. All operations
were done at atmospheric pressure and room temperature.

Determination of Carbon Dioxide Production and Oxygen Uptake

The rates of carbon dioxide production and oxygen
uptake were measured manometrically (53) at 25°C. One ml.
of the particulate fractions suspended in the appropriate
reaction mixture was used, with single and differential
respirometers and 15 ml. warburg vessels. Filter paper
soaked with 0.2 ml. of 20% potassium hydroxide in the central
well served to absorb carbon dioxide.

Determination of Total Nitrogen

The total nitrogen content of the particulate
fractions suspended in the reaction mixture was determined
by the microKjeldahl method (54).

Two ml. of the particulate suspension was first
dried at 80°C. under reduced pressure to facilitate
digestion. The digestion mixture consisted of 2 ml. of
sulphosalicylic acid (5% w/v), 1 ml. of perchloric acid
(60%, A.R.), 300 mg. of sodium thiosulphate and a drop of
saturated solution of copper sulphate as catalyst. The
digest was heated for at least two hours after clarification.

Ammonia was distilled in a Parnas-Wagner
microdistillation unit. The distillate was collected in
N/100 sulphuric acid, containing a drop of methyl red as

indicator. The method was standardised with pure ammonium sulphate and found reproducible to a lower limit of 50 µg nitrogen. Blank determinations were made each time, and samples were done in duplicate.

Determination of Inorganic Phosphorus

Inorganic (ortho) phosphorus in the particulate suspension was determined by the method of King (55).

Proteins were first precipitated in the particulate suspension) with an equal volume of 20% trichloroacetic acid and separated by centrifugation. To aliquots of the supernatant, 1.2 ml. of 60% perchloric acid (A.R.) and 1 ml. of 5% ammonium molybdate solution was added. Colour was developed with 1 ml. of amino-naptholsulphonic acid reagent. The colour was allowed to develop for 15 minutes at room temperature and read in a spectrophotometer (Beckman Model B) at 660 mµ, using a 1 cm. cuvette. Suitable standards and blanks were done each time. The method was found reproducible between 10-100 µg phosphorus. The sulphonic acid reagent was prepared by dissolving A.R. grades of 1-amino-2-napthol-4 sulphonic acid (0.5 gm.) sodium bisulphite (30 g.) and sodium sulphite (6 gm.) in 250 ml. of demineralized water. The reagent was filtered, stored in the dark at 2-5°C. and used within two weeks of its preparation.

Determination of Rate of Phosphorus Esterification

Radioactive phosphorus was used to determine the rate of phosphorus esterification (56). Sufficient $H_3P^{32}O_4$

solution was mixed with the particulate suspension to yield 60,000-80,000 cpm. activity per ml. of the reaction mixture. The reaction mixture was incubated at 25°C. with constant shaking. At different time intervals, 1 ml. samples were withdrawn and added to 1 ml. of 20% trichloroacetic acid contained in a centrifuge tube in an ice bath. The protein precipitate was rejected after 2-3 washings with 10% trichloroacetic acid and subsequent centrifugation. The supernatant and washings were pooled and a small aliquot was taken for determination of total radioactivity.

To the remaining supernatant, sufficient 15M NH_4OH was added to make it 1.5M with respect to NH_4OH. The inorganic phosphorus was precipitated as $MgNH_4PO_4 \cdot 6 H_2O$ by adding excess of magnesia mixture. The magnesia mixture consisted of 55 gm. $MgCl_2 \cdot 6 H_2O$, and 100 gm. NH_4Cl dissolved in 1.5M NH_4OH (53). After two hours of precipitation time in the cold, the precipitate was centrifuged out and washed twice with dilute ammonium hydroxide. The total radioactivity in the washings and supernatant was determined.

Radioactivity determinations were made by standard procedures using a Geiger tube (Radiation detector D37-A) of 1.4 mg./cm.^{2} window thickness. The results of radioactivity determinations were corrected for non-enzymic P^{32} exchange, background, statistical errors and other standard corrections.

Results were expressed as micromoles of phosphorus esterified per mg. nitrogen during reaction periods of 0-20 minutes.

Yield of Particulate Fractions

The weight of particulate fractions in the reaction mixture was determined by drying an aliquot of the mixture at 50°C. under reduced pressure to a constant weight. Results were corrected for the weight contributed by the substrate-buffer constituents.

RESULTS AND DISCUSSION

Section I. Performance of the Gas Chromatographic Unit as Affected by Structural Features and Operating Conditions.

Many structural features of the detector were found critical for optimum sensitivity and overall electrical stability. All electrical insulation had to be of a very high order. Plastic aluminum cement on metal to glass joints was found to be a very good insulator. The piece of fine copper mesh placed below the flames not only acted as a draught screen, but also prevented deterioration of the insulators due to deposition of combustion products.

An important setting in the detector was found to be the distance between the positive terminal and the negative, in other words, the position of the brass mesh in relation to the tip of the flame. This setting affects the sensitivity of the detector, base line stability, and pen response, particularly when the signal has been withdrawn. We got good results by keeping the brass mesh just touching the tip of the flame (about 1 cm. distance), as seen under total darkness. Furthermore, it is important that the mesh be so designed that it has no constriction in the region above the flame. The useful life of this brass mesh, as evidenced by disturbances approaching thermionic noise, is only a couple of months.

McWilliam and Dewar (51,57) developed the detector based on the principle of measuring the ion current in a hydrogen flame. They proposed the use of selective ionization utilizing the difference between the ionization potentials of the materials to be detected. Organic molecules have ionization potentials between 9-12 ev, as compared with 15.5 for nitrogen and 15.6 for hydrogen. The main advantage in using the two carrier gases in the flame as a course of excitation for selective ionization is the high signal to background ratio. In addition, the response for simple organic molecules is roughly proportional to the concentration of the molecule as well as to the number of carbon atoms in the molecule. We found the detector extremely sensitive to the concentration of hydrogen in the carrier gas and investigated the effects of different concentrations of hydrogen in the carrier gas mixture on the sensitivity of the detector. The response of the detector to various concentrations of ethylene under different flow rates of hydrogen in the carrier gas is plotted in Figure V.

The term "sensitivity" has been defined on an operational basis to include: high response of the detector, quick return of the recorder pen to the base line when the signal is withdrawn, a large ratio of signal to background, linearity of response, reproducibility, and compatible column efficiency. We could fulfill these
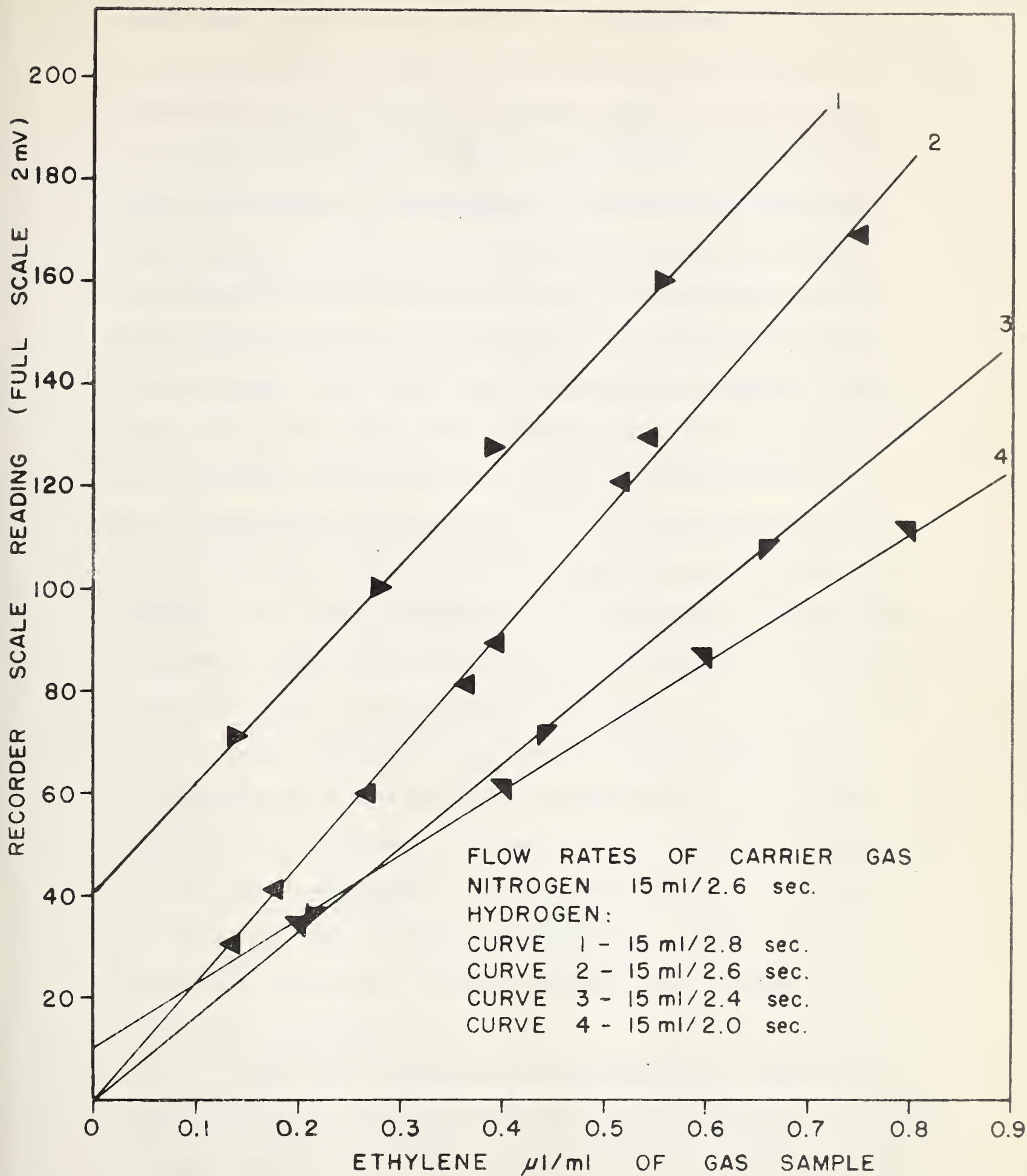
FIGURE V.

RESPONSE OF HYDROGEN FLAME IONIZATION DETECTOR TO
DIFFERENT FLOW RATES OF HYDROGEN IN THE CARRIER GAS.

requirements for sensitivity under the different

concentrations of hydrogen in the carrier gas (Figure V).

With flow rates of hydrogen varying from 2 to 2.8 seconds

per 15 ml., the retention time for ethylene varied between

22 to 30 seconds. No spreading or tailing was observed.

From Figure V, curve 2, it will be noticed that maximum

sensitivity and linearity in response of the detector has

been obtained by using a mixture of nitrogen and hydrogen

each adjusted to a flow rate of 15 ml./2.6 seconds. Under

these flow rates, the total pen response time is from 5

to 10 seconds, depending upon ethylene concentration.

Hence, by using a chart speed of 1/3 inch per minute, it

was possible to relate ethylene concentration to peak

heights. At other concentrations of hydrogen, although the

response of the detector is linear, the curves 1, 3 and 4

(Figure V) fail to go through the origin.

The detector is not particularly sensitive to

low amounts (5-10 μl) of carbon dioxide and 1 ml. of air.

Moreover, its response to carbon dioxide and air is negative

in relation to ethylene, and the emergence time is 40-50

seconds after the emergence of ethylene. Hence, it was

possible to introduce different samples for analysis at

one minute intervals.

Both, the detector and the amplifier, possessed

a high degree of linearity between a wide range (0.5 to 5 mV)

of input and output signals. At constant voltages and over

extended periods of time, both the noise level and short term drifts were within 0.01 mV, at the highest limit of sensitivity. However, sufficient warming up time to achieve thermal and other equilibrium is needed. At any particular setting, the response of the detector and amplifier is reproducible. As a routine procedure, the gas chromatographic unit was calibrated against known concentrations of ethylene, prior to each analysis.

The entire gas chromatographic procedure had all the desirable characteristics: simplicity and ease of operation, sensitivity and quantitative reproducibility. It is inexpensive, and simple to construct.

Section II. Efficiency of Ethylene Collection, and its

Quantitative Analysis.

Many methods have been used to collect and
estimate ethylene from a flowing stream of air containing
respiratory volatiles of fruits. Early investigators (4)
attempted preparations of dibromide derivatives of
ethylene and paper sensitized by red selenium (7). Hansen
and Christensen (58) have described a quantitative
microbromination technique for the analysis of ethylene.
Young, Pratt and Biale (34) replaced bromine with mercuric
perchlorate solutions to absorb ethylene and developed a
manometric procedure for quantitative estimations. Hall (27)
used direct absorption of respiratory volatiles in potassium
permanganate solutions. Thompson (59) and Meigh (60,61,62)
have used cold traps of liquid nitrogen and oxygen to
collect ethylene and other fruit emanations and subsequently
analysed them by gas chromatographic procedures. Huelin
and Kenett (63) combined mercuric perchlorate absorption
and mass spectrometric analysis to investigate the olefines
produced by apples. It would be appropriate to mention
that bio-assay techniques for ethylene have also been
described (64,65).

Spencer (46) has described a micro-collection
apparatus for ethylene and has used it for determining the
rate of ethylene production from mitochondrial suspensions
of tomato. Burg and Burg (39) did not employ a collection

device for ethylene produced by particulate fractions of tomato. They analysed the total respiratory volatiles by direct gas chromatography. Meigh et al. (36) and Lieberman and Craft (37) have not described the technique used for collection of ethylene from the respiratory volatiles of sub-cellular fractions of tomato and apple. Despite the divergence in techniques for collection and concentration of ethylene, all the investigators (35,36,37,39) of ethylene production by cell free systems have now turned to the same sensitive technique for the estimation of ethylene, that is, a gas chromatographic unit with either a katharometer or hydrogen flame ionization detector.

One of the major difficulties in the investigation of ethylene production in cell-free system, is the low rate of gas emanation. It is obvious that in such investigations the efficiency of collection should be very high, the analytical procedure quantitative and the possibility of contamination none. These aspects of our procedure are discussed below.

Collection Efficiency

With the micro-collection apparatus, Spencer (46) obtained 100% recoveries of ethylene for amounts as low as 10 μl under conditions which simulated biological evolution from kilogram quantities of fruits. At lower concentrations, the efficiency could not be satisfactorily checked, because of the limits of the sensitivity of the micromanometric procedure.

In later work, by a gas chromatographic procedure, the efficiency of the micro-collection apparatus, used in the present investigation was tested.

The two most important factors affecting the collection efficiency were found to be the flow rate and the nature of the mercuric perchlorate absorption column. Results of some of the many experiments on the recovery of very low amounts of ethylene under different flow rates and conditions of the absorption column are summarised in Table 1.

One of the limitations in such recovery experiments lies in the ability to simulate conditions of ethylene production from cell-free systems. However, the gas was regenerated at rates close to those of biological evolution (46). Two types of filter sticks, medium porosity glass filter sticks and alundum disc filters have been used in the recovery experiments. Although the glass filter sticks were commercial products, we noticed that they were not always alike in their dispersion properties, as will be seen (Table 1) from the variations in the length of foam column produced by them. It may be recalled that the absorption tube contained 4.5 ml. of mercuric perchlorate solution with 5 μl of n-butanol as a foaming agent. It is evident from the results in Table 1 that a long column of finely foaming mercuric perchlorate was of prime importance in determining the efficiency of the ethylene absorption at

TABLE 1

Effect of Flow Rates and Nature of Absorption

Column on Efficiency of Ethylene Collection

Exp. No.	Filter Stick	Flow Rate ml./min.	Absorption Column			
			Nature of Dispersion	Length (cm.)	Ethylene Added (μl)	Ethylene Recovery[*] (%)
1	Glass	55	Coarse	13-14	35	54
2	Glass	40	Coarse	12-13	22	100
3	Glass	35	Coarse	12-13	20	100
4	Glass	30	Coarse	< 10	15	60
5	Glass	35	Coarse	< 10	0.67	0
6	Glass	30	Coarse	< 10	1.24	0
7	Glass	26	Coarse	< 5	1.24	39
8	Glass	26	Coarse	< 5	0.67	34
9	Alundum	26	Fine	10-11	1.24	107
10	Alundum	26	Fine	14-15	1.24	104
11	Alundum	26	Fine	14-15	0.62	100
12	Alundum	26	Fine	11-12	0.62	100
13	Medium/ Alundum[*]	5/20[*]	Fine	10-11	0.62	100
14	Medium/ Alundum	5/20	Fine	11-12	0.62	100
15	Medium/ Alundum	5/20	Fine	11-13	0.62	100
16	Medium/ Alundum	5/20	Fine	12-13	1.24	101
17	Medium/ Alundum	5/20	Fine	10-11	1.24	100

[*]Experiment 1 to 4, ethylene determined by Manometric technique,

and in Experiments 5 to 17, ethylene determined by gas chromatography.

Oblique bars signify the combination of filters and the respective

flow rates.

very low concentrations of the gas. We found that the
medium and fine porosity glass filter sticks gave a much
coarser dispersion than the medium porosity alundum disc
filters.

The length of the foam column also depends
considerably on the flow rate. As seen from the results
in Table 1, at flow rates of 35-40 ml./minute (Ext. 2,3)
when the foam column is 12-13 cm. the recovery is 100%
when the flow rate is lowered to 30 ml./minute (Exp. 4)
there is a corresponding decrease in foam column and also
in ethylene recovery. If the length of the foam column
is increased by increasing the flow rate to 55 ml./minute
(Exp. 1) the recovery is again reduced to 54%. At lower
concentrations of ethylene and low flow rates (Exp. 5,6)
no ethylene could be recovered when glass filter sticks
were used. With 26 ml./minute flow rate (Exp. 7,8) the
recovery was only 34-39%. That is, the flow rate had to
be fast enough to create sufficiently long foam column,
but slow enough to allow absorption of ethylene. In view
of these results, we tested the efficiency of ethylene
collection using an alundum disc filters. We used "medium"
alundum porosity which is much finer than medium or fine
porosity glass. At low flow rates (26 ml./minute) these
gave very fine dispersion, sufficient to support a 10-15
cm. foam column. At low concentrations of ethylene, the
collection efficiency was 100% (Exp. 9-12) with these filters.

It may be pointed out that in recovery experiments
(Exp. 1-12) where the glass filter sticks or the alundum
disc filters have been used singly, the total gas flow
was allowed to pass through the reaction vessel. These
experiments (Exp. 1-12) suggested that for 100% collection
efficiency for low concentrations of ethylene, the
absorption tube should have a 10-13 cm. column of very
finely foaming mercuric perchlorate solution. Increasing
or decreasing the flow rates adversely affects the nature
and height of the foam column and consequently the collection
efficiency. All results indicate the need for a critical
combination of flow rate and a finely foaming column of
mercuric perchlorate solution. The foaming property of
the mercuric perchlorate can be improved by increasing
the concentration (10-20 μl) of n-butanol. However,
increasing concentration of n-butanol was found to affect
adversely the subsequent regeneration of the gas.

The use of alundum disc filters in the collection
assembly had the serious disadvantage of building up
pressure greater than atmospheric inside the reaction
vessel. In view of this limitation, the absorption tube
was fitted with an alundum disc filter connected to an
auxiliary air supply, flowing at the rate of 20 ml./minute.
Its purpose was to maintain a 10-13 cm. column of finely
foaming mercuric perchlorate solution. The reaction vessel
was connected to the absorption tube through the medium

porosity glass filter sticks carrying air supply at the
rate of 5 ml./minute. With such a combination it was
found easy to maintain a steady 10-13 cm. column of finely
foaming mercuric perchlorate solution without altering
either the total flow rate (25 ml./minute) inside the a
absorption tube or the concentration of n-butanol. To
achieve these critical settings, it is essential to place
the two filters close to each other. Under these rigid
conditions, 100% recoveries of low concentrations of
ethylene were obtained (Exp. 13-17). The alternative
collection assembly (page 21) using a liquid nitrogen-cooled
trap with air as the carrier (10 ml./minute) was not found
quantitatively satisfactory, because of large amounts of
condensation of air in the collection trap, and insufficient
temperature differential between the temperature of liquid
nitrogen and the boiling point of ethylene.

Regeneration of Ethylene

We compared our method of liberation of ethylene
from the ethylene mercuric complex with the procedure
described by Burg (30). Our gas chromatographic procedure
was used to check recoveries in the concentration range of
1-5 μl ethylene. Using hot or cold ammonium sulphate
solution (5%) as the refluxing agent in Burg's apparatus,
we recorded only 30-50% recoveries, as compared with 100%
by our own method, described on page 21.

Introduction of Sample to the Gas Chromatography Apparatus

The Hamilton gas tight syringe used to inject gas samples into the analytical column had a teflon plunger and required no sealing grease. These syringes, when used dry, were found to give fully reproducible results as compared to ordinary hypodermic syringes, where the glass plungers require grease seals.

We also compared the performance of the Hamilton syringe with the by-pass device described by Meigh (50) to introduce gas samples into the column. While the two methods were found comparable, our method of injecting gas samples had the advantage of ease of operation.

From the above results, it was thus concluded that in our analytical procedure 100% of collection of ethylene was achieved, and liberation of the gas and its injection into the gas chromatographic column were both quantitative and fully reproducible even at very low concentrations of ethylene. An additional advantage was the ease of operation, of special value when a large number of samples have to be analysed at one time.

Section III. Identification of Ethylene

In our earlier work (35) we used a micromanometric procedure (46) for analysis of ethylene. However, with the amounts of ethylene obtained from cell-free systems, we were working at the limits of accuracy of this procedure, and it was obvious that for quantitative comparisons we would have to turn to a more sensitive procedure such as flame ionization detection. The manometric analysis, which is specific for olefinic hydrocarbons (34), and mass spectrographic analysis have been used in our experiments to provide qualitative corroborating evidence to that from the gas chromatograph   that we are, in fact, dealing with ethylene. Moreover, negative results for ethylene in mercuric perchlorate solutions, aerated at the rate of 25 mls. per minute for 4 to 16 hours in our collection assembly, eliminated all possibility of contamination. In none of our gas chromatographic and mass spectrographic analyses have we found evidence for the presence of any olefinic volatile besides ethylene. It should be noted the gas chromatographic analyses for ethylene were made on gas collected in mercuric perchlorate solution and liberated by addition of lithium chloride, a procedure specific for olefinic hydrocarbons. Burg and Burg (39), on the other hand, injected the complete mixture of gases evolved by particles directly into the gas chromatograph. Moreover, they found that the "ethylene-like" substance of

Lieberman and Craft was not absorbed by mercuric perchlorate,
although authentic ethylene was. (Neither Meigh and
co-workers (36) nor Lieberman and Craft (37) have stated
either the method or time they used for ethylene collection).
It is therefore evident that in our analysis we are dealing
with a true olefinic hydrocarbon, and not with the "ethylene-
like substance" noted by Burg and Burg (39). Moreover,
these authors did detect minute amounts of true ethylene
from their acidic particles, although they were prepared
under very different conditions from those we had reported.
As further indication that we were in fact dealing with
ethylene, mass spectrographic analysis showed that the gas
collected by our technique exhibited peaks characteristic
of ethylene. Further confirmation was obtained by
co-chromatography with known ethylene and disappearance of
chromatographic peak when the gas was passed through columns
of brominated activated carbon. Huelin and Kennett (63)
showed that normal olefins from propylene to hexene were
not present in proportions exceeding one part of higher
olefin to 1,000 parts of ethylene in apple volatiles
collected in mercuric perchlorate. They indicated that
branched chain or multi-unsaturated hydrocarbons would not
be regenerated with ethylene from the mercuric perchlorate
solution on addition of lithium chloride, and they could
find no evidence of saturated hydrocarbons.

Section IV. Comments on the Preparative Procedure for,
and the Nomenclature of, the Sub-Cellular Fractions of Tomato.

Differential centrifugation techniques have become
standard preparative procedure for the isolation of particulate
fractions from cells of all organisms. However, it is
doubtful if any of the fractions prepared by differential
centrifugation, even with washings and recentrifugations,
can be regarded as approaching homogeneity (66,67,68).
There appears to be a real need for improved techniques
for purification of fractions and evaluation of structural
and functional relationships of particulate systems (68).

Much of the difficulty in the separation and
characterization of sub-cellular structures from plants is
basically due to inadequacy of knowledge of size variations
of mitochondria and microsomes in plant cells (66,69,70).
Not only do the sizes of sub-cellular structures vary among
organisms (pea mitochondria, for instance, being much
smaller than liver mitochondria), but variation with age has
been suggested in the report that mitochondria disintegrate
as fruits ripen (71).

Spencer (35) outlined a preparative procedure for
the isolation of mitochondrial particles active in ethylene
production. In the present investigation, we have followed
essentially the same scheme (Figure I) extending it to the
isolation of several sub-cellular fractions from tomatoes.
As a routine procedure, the cell fragments-chloroplast-nuclei

fraction was obtained at 4,000g and "mitochondria" at
35,000g. The mitochondrial particles could be separated
at 15,700g, but centrifugation at 22,000g and 35,000g
separated additional particles active in ethylene production
(Table 2). Since the sum total of ethylene production by
these three fractions equalled that of the 35,000g fraction,
it was considered advantageous to isolate a fraction at
35,000g as a routine procedure. An added advantage was that
higher centrifugal force packed the particles into easily
handled pellets.

Between 35,000g and 40,210g, we were able to
separate a fraction active in ethylene production. Both
the 35,000g and 40,210g fractions could be stained with
Janus Green B, but quantitative aspects of staining could
not be assessed with the available microscopes. It is
customary to isolate "microsomal" particles from supposed
mitochondria-free supernatants by centrifugations at 100,000g.
The "microsomal" preparations have been shown to be
biochemically heterogenous systems consisting of membrane
bound vesicles, membrane fragments, and small spherical
particles 100-300 A° in diameter, both free and attached
to membranes. It has been often suggested that they are
probably fragments of endoplasmic reticulum (67). In view
of the above, we contend that the 40,210g fraction, obtained
after the separation of the 35,000g fraction, is a sediment
containing fragmented mitochondria enriched with fragments

TABLE 2

Comparison of Ethylene Production by Sub-Cellular Fractions from

Tomatoes, Sedimented at Different Centrifugal Forces*

(μl x 10^3/mg.N)

Physical State	Time	Average Centrifugal Forces				
		15,710g (15 min.)	22,620g (15 min.)	35,000g (15 min.)	40,210g (15 min.)	105,000g (10 min.)
Intact	0-4 hrs.	0	0	-	21.9	-
	4-16	Trace	23.3	Trace	31.4	-
Sonically	0-4	47.5	29.6	64.9	71.3	57.4
Treated	4-16	11.2	16.0	48.8	73.8	160.4

Reaction mixture: 0.5M sucrose, 0.125M KH_2PO_4, 10^{-3}M $MgSO_4$, 10^{-3}M $MnSO_4$, 0.05M L-malic acid, adjusted to pH 7.0 with sodium hydroxide. This mixture was sterilized prior to use; 1.98 x 10^{-3}M adenosine triphosphate (ATP) and 3.3 x 10^{-4}M diphosphopyridine nucleotide (DPN) were added after sterilization. 25°C.

*Cell fragments, nuclei and chloroplasts were removed by a preliminary centrifugation at 4,000g for 7 minutes.

of endoplasmic reticulum, while the 105,000g fraction represents the remaining "microsomes".

The object behind direct isolation of the 40,210g fraction from the supernatant of 4,000g centrifugation was to prepare a cell free system which might be expected to be more active than either of the consecutively separated 35,000g and 40,210g fractions alone. It is obvious that this combined fraction contained mitochondria, and fragments of mitochondria, and endoplasmic reticulum.

When, according to the accepted procedure, tissues are initially ground in a sucrose-buffer medium, not only the whole cells but also some sub-cellular particles are likely to be disrupted. In addition to these mechanical effects, osmotic effects must be considered. Zeigler and Linnane (72) and others (68) have shown that the structural integrity (and hence the biochemical activity) of mitochondria is dependent on the osmotic environment. This was borne out in our preparative procedure, when we found that the supernatnat from the centrifugation at 40,210g was much more active in ethylene production when particles were prepared in 0.25M sucrose rather than 0.5M sucrose. This was our first clue that the activity of the ethylene-producing system was in some way related to the structure (or structural integrity) of the particles. Equipped with this knowledge, further investigations on the relation between the physical state of the particles and ethylene production were conducted.

A further critical point in obtaining active
fractions lies in the method of washing and suspending
them in the buffer substrate mixture. In both these
steps, in our earlier work, according to common practice,
we used a Servall "Omnimixer" at very low speeds (25-30 V
on the powerstat) for 1-2 minutes in the cold. Omission
of the Omnimixer washing of the particles and use of the
instrument only to suspend them in the final reaction
mixture gave particulate fractions more active in ethylene
production. Very gentle washing of the particles with the
help of a glass rod to disperse them, had no measurable
effect on ethylene production. In the washings, by use of
the Omnimixer, some fragmentation of particles inevitably
occurred and upon recentrifugation, the fragments remained
in the supernatant and were discarded. Later work showed
that these fragments were primarily responsible for the
initial ethylene production. Some results indicating the
consequence of washing procedure on ethylene production are
shown in Table 3. As will be seen, Omnimixer washing results
in detectable ethylene production in the supernatant while
gentle washing with a glass rod had no such adverse effects.

Since nitrogen determinations were not done on
these preparations, the results have been expressed as
microliters of ethylene produced in 4 hours from particles
originally isolated from 900 gm. of tomato. A total volume
of 90-95 ml. of buffer (0.5M sucrose-0.01M KH_2PO_4, adjusted

TABLE 3

Effect of Washing Procedures on Ethylene Production

by Particulate Fractions of Tomato

Fractions	Washing Procedure	Ethylene (μl/4 hr.)	
		Particle	Washings
Cell Fragments: Chloroplast:Nuclei	Omnimixer	0.2363	0.1260
	Glass Rod	0.0945	Absent
Mitochondria	Omnimixer	0.0606	0.0200
	Glass Rod	0.1060	Absent
"Fragmented Mitochondria: Microsomes"	Omnimixer	0.0550	Trace
	Glass Rod	0.1500	Not done
"Combined Mitochondria: Microsomes"	Omnimixer	0.0393	Trace
	Glass Rod	0.0788	Absent

Reaction mixture as in Table 2.

to pH 7.0 with NaOH) was used for washing each particulate
fractions. The particulate fractions were recovered by
recentrifugation at the appropriate speed and suspended
in 25 ml. of reaction mixture (page 53) with the help of
an Omnimixer. Hence, these preparations are to be considered
as partially disintegrated systems. The rates of gas
production in the different particulate fractions are not
strictly comparable, since these results have been
summarized from different experimental runs. However, they
definitely show that the washing with the Omnimixer is
detrimental and question the very usefulness of the
washing procedure as far as ethylene production is concerned.
Consequently, in our later work, we omitted the washing step
from the preparative procedure, in order to obtain more
active preparations. An added advantage was a 2 hour
reduction of _in vitro_ aging of the preparations, some
consequences of which are pointed out in Section VI.

In the text, we have chosen to designate the
particulate preparations as intact, sonically treated, and
partially disintegrated systems. While there can be no
objection to the nomenclature of the sonically treated
preparations, critics may prefer to designate our intact
fractions as untreated. The fact that these preparations
show a typical lag period in carbon dioxide production, a
behaviour characteristic of morphologically and structurally
integrated mitochondria (68,72), justifies the designation

as "intact". As pointed out earlier, "partially disintegrated"
preparations were obtained by 1-2 minutes treatment at low
speeds of the Servall "Omnimixer". It is our experience
that this and other such instruments (e.g. the Virtis "45"
homogenizer), even with the most careful use, tend to
partially fragment the particulate preparations. In such
treatments, a certain degree of protein denaturation could
also be expected. It is obvious that such mechanical
treatments are not very reproducible. In view of these
considerations, where the "Omnimixer" was used at very low
speeds (20-30 V on the powerstat) at ice temperatures for
1-2 minutes, the preparations were designated as "partially
disintegrated systems".

As pointed out earlier, we always prepared the
homogenate from 850-900 gms. of fruits. Some steps in the
preparative proceudre (crushing and pressing the frozen
tomatoes to prepare the homogenate) could not be conducted
strictly quantitatively. Hence the yield of the particulate
fractions varied considerably among runs. In 12 runs the
yield of mitochondria varied from 175 to 190 mg. per
kilogram of tomatoes, that of the "fragmented mitochondria:
microsomes" (40,210g) from 62-82 mg. per kilogram and the
"combined mitochondria-microsome" fraction from 149 to 205
mg. per kilogram.

Section V. Biochemical Properties of the Sub-Cellular
Fractions of Tomato Active in Ethylene Production.

With the discovery that mitochondria are an
active site of ethylene production (35), these investigations
were undertaken to study some of the biochemical properties
of the different sub-cellular fractions of tomato that are
active in ethylene production. In these studies, among
our earliest, the particulate fractions were initially
ground in 0.25M sucrose-0.5M potassium dihydrogen phosphate
buffer medium of pH 8.0. The final pH of the homogenate
was 7.0. The particulate fractions were both washed and
suspended in the reaction mixture with the help of the
"Omnimixer".

Representative results of the determination of
nitrogen content, rates of phosphorus esterification, and
carbon dioxide and ethylene production are summarized in
Tables 4-6. Each experiment in Tables 4-6 represents the
values from two or more experimental runs which were
strictly comparable. Ethylene was collected for four hours,
and carbon dioxide evolution was determined for half-hour
periods at the beginning and end of the ethylene collection
time. The rate of esterification was followed for a
reaction period of 0-20 minutes. In the absence of a steady
state of phosphorus esterification, the + sign values have
been used to indicate if there was significant uptake of
labelled phosphorus during the 0-20 minute reaction periods.

TABLE 4

Biochemical Properties of Mitochondria[**] from Tomatoes

Run No.	Nitrogen (μg/mg.)	Carbon dioxide (μl/mg N/1/2 hr.)		Esteri-fication 0-20 mins.	Ethylene 4 hrs.	Technique for Ethylene Analysis
		Beginning	End			
1	40	49	57	*	+	Manometric
2	50	58	63	*	+	Manometric
3	57	51	71	*	+	Manometric
4	46	40	*	+	+	Gas Chromatographic
5	*	41	*	+	+	Gas Chromatographic
6	*	53	*	+	+	Mass Spectrometric
7	*	53	*	+	+	Gas Chromatographic
8	*	46	*	+	+	Mass Spectrometric

* not done

+ detected

** Separated at 35,000g/15 minutes from the supernatant of the 4,000g fraction. The reaction mixture consisted of 0.5M sucrose, 0.125M KH_2PO_4, 10^{-3}M $MgSO_4 \cdot 7H_2O$, 10^{-3}M $MnSO_4 \cdot 4H_2O$, 0.25M l-malic acid, 1.98 x 10^{-3}M ATP, 3.3 x 10^{-4}M DPN pH 7.0, 25°C.

Mass spectrometric and gas chromatographic analysis of
ethylene was done on a qualitative basis. Micromanometric
procedures indicated that we were working between 5 and 8 μl
of ethylene, which is the limit of sensitivity of this
procedure. Hence these values have been expressed
qualitatively, and simply as corroborating evidence for
the production of ethylene. From the results (Table 4),
it is evident, first of all, that there was considerable
variation (40-50 μg/mg.) in the nitrogen content of the
mitochondrial fraction. Variations (40-58 μl/mg N/1/2 hour)
are also seen in the carbon dioxide production. In all
cases there is a tendency for increased carbon dioxide
production (57-71 μl/mg N/1/2 hour) at the end of the
ethylene collection period. In some cases, the P:O ratios
did work out to 2.4-2.7, but in the absence of a steady
state of esterification, these values could not be accepted
as quantitatively meaningful. It may be emphasized that
use of either M/100 sodium fluoride (an inhibitor of ATPase)
or M/100 dinitrophenol (a phosphorylation uncoupling agent)
had no measurable effect on any of the determinations.

The "fragmented mitochondria:microsomes" fraction
represents the particles sedimented at 40,210g/15 minutes
after the separation of the 35,000g/15 minutes fraction.
As with the mitochondria, the results with "fragmented
mitochondria:microsomes" (Table 5) indicate considerable
variation in the nitrogen content (90-110 μg/mg.) with

TABLE 5

Biochemical Properties of "Fragmented
Mitochondria:Microsomes"[**] from Tomatoes

Run No.	Nitrogen (µg/mg.)	Carbon dioxide (µl/mg N/1/2 hr.)		Esteri-fication 0-20 mins.	Ethylene 4 hrs.	Technique for Ethylene Analysis
		Beginning	End			
1	90	47	*	*	+	Manometric
2	87	43	64	*	+	Manometric
3	110	52	64	*	+	Manometric
4	95	50	70	+	+	Gas Chromatographic
5	*	*	*	+	+	Gas Chromatographic
6	*	*	*	+	+	Mass Spectrometric
7	*	*	*	+	+	Gas Chromatographic
8	*	*	*	+	+	Mass Spectrometric

[*] not done

[+] detected

[**] Particles were separated by centrifugation of the 35,000g
supernatant at 40,210 x g for 15 minutes.

increased carbon dioxide evolution at the end of the
ethylene collection period. The preparations showed
evidence of esterification of phosphorus as well as
ethylene production. Use of M/100 NaF and DNP had no
measurable effect on any of the determinations. Similar
properties of this fraction and the mitochondria lend
support to the suggestion that this fraction, active in
ethylene production, contains fragmented mitochondria.

The object in isolating the two fractions
together was to obtain a cell-free system more active in
ethylene production than either fraction separately. It
will be seen (Table 6) that there is a wide variation in
the nitrogen content (60-130 μg/mg.) of this fraction.
As compared to either mitochondria (Table 4) or the
fragmented mitochondria:microsomes (Table 5), the rate
of carbon dioxide production in this preparation is
slightly less in the beginning of ethylene collection and
exhibits a similar tendency towards increased carbon
dioxide production at the end of the ethylene collection
period. Although esterification was evident, surprisingly
no ethylene production could be detected. It must be
pointed out that supplying additional co-factors
(5×10^{-4} adenosine diphosphate, 0.3×10^{-6} triphosphopyridine
nucleotide) or the addition of NaF (M/100) or DNP (M/100)
had no measurable effect on any of the measured properties.

TABLE 6

Biochemical Properties of the "Combined

Mitochondria:Microsomes"[**] from Tomatoes

Run No.	Nitrogen (μg/mg.)	Carbon dioxide (μg/mg N/1/2 hr.)		Esteri- fication 0-20 mins.	Ethylene 4 hrs.	Technique for Ethylene Analysis
		Beginning	End			
1	60	28	38	+	−	Manometric
2	130	33	62	+	−	Manometric
3	130	45	62	+	−	Manometric
4	*	33	*	+	−	Mass Spectro-metric
5	80	33	*	+	−	Gas Chroma-tographic

[*] not done

+ detected

− not detected

[**] Particles were separated by centrifugation at 40,210g/45

minutes of the supernatant from the separation performed

at 4,000g.

Thus, all the fractions (Tables 4,5,6) showed considerable variations of their biochemical properties within themselves. These results bear out the point stressed: namely that available methods for separation of sub-cellular constituents cannot be considered as quantitative or as yielding homogeneous fractions. It may be recalled that in all these experiments, the particulate fractions were washed and the washings rejected. As revealed later, many active ethylene-producing fractions were thus discarded. In addition, since they were subjected to the Omnimixer, all these systems have to be looked upon as partially disintegrated systems which could be expected to be active in ethylene production. The reasons for the lack of detectable ethylene production in the "combined mitochondria microsome" fraction became apparent in our later work, and are discussed in conjunction with it (page 68). The results do not permit any valid conclusions concerning the possible relationship between ethylene production, phosphorylation, or carbon dioxide production.

Section VI. Relation of Ethylene Production to the Physical
State of the Sub-Cellular Fractions from Tomato Fruit.

The positive identification of ethylene and the
development of quantitative, sensitive analytical procedures
made possible a study of the biochemistry of ethylene
production. Our studies on the biochemical properties of
the fractions active in ethylene evolution (Section V) and
on the critical aspects of the preparative procedure
(Section IV) showed that the ethylene-producing system
was located in the sub-cellular fraction. Moreover, there
were indications that the activity of the system was in
some way linked to the degree of structural integrity of
the particles. All results pointed to the need for a
critical study of the relation of ethylene production to
the physical state of the sub-cellular particles.

In the present study, the particulate fractions
were isolated in the 0.5M sucrose-0.5M KH_2PO_4, pH 8.0
buffer medium. The particulate fractions were not washed
but were directly suspended in the reaction mixture of the
composition indicated in Table 7. "Intact", "sonically
treated" and "partially disintegrated" fractions were
prepared by the methods outlined on page 16-17. Ethylene
was collected for different time intervals and quantitatively
determined by our gas chromatographic procedure. The
results are summarized in Tables 7-11. It will be seen
(Table 7) that intact mitochondria did not produce detectable

TABLE 7

Ethylene Production by Mitochondria

(μl x 10^3/mg.N)

Physical State	Time	Run No.: 1	2	3	4	5	6	7
Intact	0-4 hrs.	0	0	0	0	0	-	-
	4-8	6.3	14.7	-	-	-	-	-
	8-16	21.1	17.5	5.7	-	13.0	-	-
Partially	0-4	-	-	-	-	23.8	29.0	16.6
disintegrated	4-8	-	-	-	-	Trace	-	-
	8-16	-	-	-	-	3.0	29.0	29.0
Sonically	0-4	-	235	-	113	-	-	-
treated	4-8	-	17.7	-	-	-	-	-
	8-16	-	13.3	-	7.9	-	-	-

Reaction
Mixture: 0.5M sucrose, 0.125M KH_2PO_4, 0.05M l-malic acid, 10^{-3}M $MgSO_4$, 10^{-3}M $MnSO_4$, 1.98 x 10^{-3}M ATP, 3.3 x 10^{-4}M DPN; pH 7.0; 25°C.

amounts of ethylene during the first four hours, but as the
reaction time progressed, they presumably disintegrated
and ethylene evolution occurred. The partially
disintegrated mitochondria produced much of the ethylene
during the first four hours, as did the sonically treated
particles. Markedly higher ethylene production was
obtained with sonically treated particles than with the
partially disintegrated or with the intact particles. It
was interesting that, in all cases, ethylene production
continued over extended periods of time, at a slow rate.

The fraction isolated at 40,210g for 15 minutes,
when untreated, showed maximum ethylene production during
the first four hours (Table 8). Again partial disintegration
or sonic treatment resulted in considerably greater
production of ethylene. As with the mitochondria, activity
was retained for some time.

The purpose behind isolation of a combined
mitochondrial:microsomal fraction was to prepare a cell-free
system capable of still higher ethylene production. From
the results in Table 9, it will be seen that although this
preparation was active, the rate of ethylene production on
a nitrogen basis was lower than that of either the
mitochondrial or the microsomal fraction. (Sonic treatment
did appear to activate the system). While many possible
explanations for the inhibition on the basis of possible
uncoupling of oxidative phosphorylation (24,25) or the

TABLE 8

"Fragmented Mitochondria:Microsomes"

$(\mu l \times 10^3/\text{mg.N})$

Physical State	Time	Run No.: 1	2	3	4	5	6	7
Intact	0-4 hrs.	56.4	28.0	21.4	0	25.0	–	–
	4-8	13.8	1.0	↓	↓	↓	–	–
	8-16	13.8	3.8	21.4	28.0	2.5	–	–
Partially	0-4	–	–	–	–	86.7	80.7	90.0
disintegrated	4-8	–	–	–	–	Trace		
	8-16	–	–	–	–	8.6	80.7	45.0
Sonically	0-4	–	87.6	–	93.6	–	–	–
treated	4-8	–	↓	–	↓	–	–	–
	8-16	–	18.1	–	20.0	–	–	–

Reaction mixture as in Table 7

TABLE 9

Ethylene Production by Combined Mitochondria-Microsomes

$(\mu l \times 10^3/mg.N)$

Physical State	Time	Run No.:	8	9	10	11
Intact	0-4 hrs.		4.5	22.5	11.4	-
	4-16		8.0	0	-	-
Partially	0-4		-	-	-	11.2
disintegrated	4-16		-	-	-	22.6
Sonically	0-4		23.7	36.0	52.0	78.4
treated	4-16		9.0	0	-	7.1

Reaction mixture as in Table 7

presence of natural inhibitors etc., can be advanced,
further experiments under a variety of experimental
conditions need to be conducted before valid conclusions
can be reached. It is conceivable that, rather than a
simple inhibition of the ethylene producing system, the
combination of the two particles may provide an alternate,
preferred pathway for utilization of intermediates in
the ethylene-producing system, or even of ethylene itself.

The ethylene producing activity (trace amounts
to 0.01 μl/16 hrs.) of the supernatant liquid was found to
depend on the stage of ripeness of the tomatoes and on the
method of preparation of the particulate fractions. For
example, more ethylene was found when 0.25M sucrose was
used in the buffer in which the tomatoes were originally
ground than when 0.5M sucrose was used. The higher molarity
of sucrose is known to be beneficial for preservation of
the integrity of mitochondria (72), and ethylene production
by the supernatant fraction from preparations where 0.25M
sucrose was used was probably due to disrupted mitochondria.

The cell fragments-chloroplasts-nuclei fraction
(Table 10) produced ethylene in small amounts, with
stimulation of production by either partial disintegration
or sonic treatment. Thus, under the experimental conditions
used, all the sub-cellular fractions have shown ethylene-
producing activity, and maximum production was obtained with
sonically treated mitochondria.

TABLE 10

Ethylene Production by Cell Fragments-

Chloroplasts-Nuclei Fraction

($\mu l \times 10^3$/mg.N)

Physical State	Time	Run No.:	8	9	10
Intact	0-4 hrs.		3.3	-	-
	4-16		Traces	-	-
Partially	0-4		-	-	28.1
disintegrated	4-16		-	-	4.6
Sonically	0-4		67.8	12.3	-
treated	4-16		7.1	0	-

Reaction mixture as in Table 7

All fractions except the cell fragments-nuclei-chloroplasts produced carbon dioxide (43-58 μl/mg. N/1/2 hr.). Our preparations of intact mitochondria showed the typical (72) lag period of 10-15 minutes before carbon dioxide production began, but after that continued to produce it at a normal rate during the entire four-hour collection period.

In this series of experiments, sonic treatment has been done on an empirical basis. It is obvious that the efficiency of sonic treatment would depend on such factors as concentration and size of the particles in the medium, viscosity, time, and temperature. We found that 4 minute sonic treatment of the mitochondrial particles (under the same experimental conditions as in Table 7) resulted in approximately equal distribution of ethylene-producing activity between the particulate (0.0428 μl/mg N/2 hours) and soluble phase (0.0480 μl/mg N/2 hours), when separated at 35,000g/15 minutes.

Another factor to be considered in drawing comparisons is reproducibility. In all of our experiments we used the same amount of fruit and followed the outlined procedure closely. However, many steps in the procedure (for example, crushing and pressing the frozen tomatoes to prepare the homogenate) could not be conducted strictly quantitatively. While nitrogen determinations themselves were reproducible, considerable variation in nitrogen content

of the fractions from various runs occurred. Comparisons
are thus more valid when made on different fractions from
one run, rather than on the same fractions from different runs.

If the results are examined in the light of these
remarks, it will be seen that sonic treatment has alway
activated the system as compared with the intact fractions.
The same conclusions emerge if one compares (Table 11)
average values of ethylene production from different
fractions. It is clear that sonically treated mitochondria
produced the greatest amount of ethylene. From the works
of Zeigler (72) and others (68) it is known that the
biochemical properties of mitochondria vary greatly with
their state of morphological and structural integrity. In
our preparative procedure when the fruit is initially
disintegrated by a Waring blendor or similar device, many
sub-cellular particles may be fragmented, just as they are
in any further mechanical washing and dispersing procedure.
As has been pointed out, the fraction isolated at 40,210g
has to be looked upon as containing partially disintegrated
sub-cellular particles enriched with fragments of endoplasmic
reticulum. The production of ethylene in relatively large
amounts by this fraction could be due to the presence of
fragmented mitochondria. The fact that the maximum ethylene
production occurs during the first four hours, and then
tapers off is characteristic of fragmented particles. If
this is the case, it could be concluded that the ethylene-
producing system is located in the mitochondria. In Table 2,

TABLE 11

Ethylene Production by Different Sub-Cellular Fractions

(μl $\times$ 10^3/mg.N, average values)

Physical State	Time	Mitochondria	Microsomes fragmented mitochondria	Mitochondria-Microsomes	Cell fragments chloroplasts: nuclei
Intact	0-4 hrs.	0	32.7	12.8	3.3
	4-8	10.5	7.4	→	→
	8-16	14.3	13.9	4.0	Traces
Partially disintegrated	0-4	23.1	85.8	11.2	28.1
	4-8	Trace	Trace	→	→
	8-16	20.3	44.4	22.6	4.6
Sonically treated	0-4	174	90.6	47.5	4.0
	4-8	17.7	→	→	→
	8-16	10.6	19.0	5.4	3.5

Reaction mixture as in Table 7

it will be noticed that the "microsome" fraction isolated
at 105,000g for 10 minutes was active in ethylene
production. It has often been suggested (79) that with
disrupted mitochondria in the medium, about half of the
mitochondrial fragments and proteins remain in the
supernatant. While many experiments would be needed to
evaluate the purity of a "microsomal" fraction, it is
conceivable that in this particular case (Table 2), the
activity in the 105,000 fraction reflects the presence of
sub-mitochondrial fragments and proteins in the supernatant
of 40,210g separation.

It is also clear that the ethylene-producing
system is activated by disintegration of the mitochondria.
This conclusion is of interest in relation to the
climacteric rise in ethylene production in fruits, for it
has been reported (71) that mitochondria disintegrate as
fruits ripen.

From the foregoing discussion, it is clear that
the discrepancies in results from different laboratories
may well arise from the use of different techniques for
preparation of cell fractions and for collection of ethylene.

Preliminary experiments with sonically treated
mitochondria have shown that the ethylene-producing system
is heat-labile (when subjected to a temperature of 80°C.
for 7 minutes) and that the major portion (60%) of the ethylene
is produced within the first hour, after which the rate of
production falls off to trace amounts, difficult to analyse
quantitatively even at one-hour time intervals.

Section VII. Effect of Calcium ions, Cholic acid, Thiomalic Acid
and Phospholipase A from Snake Venom on Ethylene Production
by "Intact" Tomato Mitochondria.

These experiments were done to explore further
the relationship between the structural integrity of
mitochondria and their ability to produce ethylene. The
results of these experiments are assembled in Table 12.
All these results are from different experimental runs,
duplicated (indicated in Table 12 as "A" and "B") for
each treatment. In these investigations, the particles
were prepared from fully ripe tomatoes obtained from a
packing house. Ethylene collection was made for 2 and 16
hour periods and analysed by gas chromatography. The
preparation of Phospholipase A from Snake Venom has been
described on page 17.

From the results (Table 12), it is evident that
treatment of "intact" mitochondria with Ca^{++}, cholic acid,
or Snake Venom Phospholipase A, resulted in increased
ethylene production. Aging effects are also apparent in
these treatments. Thiomalic acid (treatment 5) appears to
activate the system similarly to sonic treatments (No. 2).
It may be pointed out that in treatment 5, four and twelve
hour ethylene collections were analysed. Lieberman and
Craft (37) also noticed beneficial effects of thiomalic
acid. However caution must be exercised in suggesting
thiomalic acid as a better substrate, for there was

TABLE 12

Effect of Calcium ions, Cholic Acid, Thiomalic Acid,

and Snake Venom Phospholipase A on Ethylene

Production by "Intact" Tomato Mitochondria

No.	Treatment of Mitochondria	Collection Time (hours)	Ethylene Amount ($\mu l \times 10^3$/mg N) A	B
1	Untreated	0-2	0	0
		2-18	24	22
2	Sonically treated (4 minutes)	0-2	154	140
		2-18	40	24
3	Ca^{++} (3 mM)	0-2	7.3	9.1
		2-18	16.2	27.8
4	Cholic Acid (1 mg./ml.)	0-2	7.8	10.0
		2-18	41.3	49.2
5	Thiomalic Acid (0.05M)	0-4	170	not done
		4-16	11	not done
6	Phospholipase A (approx. 1 mg./ml.)	0-2	20.4	15.6
		2-18	36.0	24.5

The reaction mixture was the same as in Table 7
in all experiments, with the exception of treatment 5, where
thiomalic acid was substituted for malic acid. The final
reaction mixture contained 0.263 to 0.302 mg. N/ml.

considerable liberation of free sulphur in the reaction mixture, as evident by the dark colouration of the reaction vessel. Valid conclusions must be deferred until the disruptive effects of sulphur compounds are better understood.

While no claim is made of the use of these reagents under optimum conditions, it is evident that they all do bring about an initial activation of the ethylene producing system.

The presence of Ca^{++} is necessary for the activity of phospholipase A (45). From Forti's work (73) on pea mitochondria it is known that Ca^{++} ions can stimulate the swelling of the mitochondrial particles. It is well known that mitochondria are rich in phospholipids, thought to be important in maintenance of structure, (68) and phospholipase A thus could be expected to destroy the structural integrity of the particles. When the results of treatment 6 and treatment 3 are compared, it is evident that addition of phospholipase A resulted in greater stimulation of ethylene production than did the addition of Ca^{++} alone. The use of detergents and other emulsifying agents to promote swelling of mitochondrial membranes has become common in studies of the structural and functional relationships of sub-cellular particles (68). Cholic acid can be assumed to extract the enzymes from the mitochondrial particles (75) resulting in a disintegrated system. It is interesting that in treatments: 3,4,6, aging has produced some activation. It is known

that aging can cause permeability changes and probably
disruption of the particulate structure (76). There is
much experimental evidence regarding the effects of such
agents on particulate structures, but it is beyond the
scope of this thesis to deal with them in detail. The
important fact which emerges is that results from the use
of these agents in our preparations support the view that
the ethylene producing system is activated when the
structural integrity of the mitochondria is destroyed.

Section VIII. Ethylene Production by Particles from Rat
Liver, Rat Intestinal Mucosa and Penicillium digitatum, and
Ethylene Content of the Respiratory Gases from Human Subjects.

Ethylene contents of respired gases from human
subjects are indicated in Table 13.

While many experiments would be needed to
evaluate the effects of sex, age, physical conditions etc.,
it is clear from the results in Table 13, that the exhaled
gases of normal human subjects are richer in ethylene than
the air inhaled by them.

A more direct approach to the question of ethylene
production by animals was to attempt in vitro preparations
capable of evolving the gas. (Method used for such
preparations are described on page 16). The results of a
few trial experiments on the production of ethylene by
fractions from rat liver and rat intestinal mucosa are
assembled in Table 14.

The lack of consistency in the level of ethylene
production by fractions from rat liver reflects the
difficulties in quantitative preparation of such fractions.
It is quite probable also that some fragmentation of these
particles occurred when the liver was originally ground
with the "Omnimixer". Sonic treatment did not increase
the ethylene producing activity of intestinal mucosa.
While further experiments with refined techniques are
needed, it is of interest that we were able to obtain

TABLE 13

Ethylene Content of Room Air and Exhalations

of Adult Human Subjects[*]

| | Ethylene | | |
Source	Gas Chromatography (ppm)	Mass Spectrometric	Remarks
Room Air	0.0067	not done	--
Subject 1	0.0090	not done	Female, Fasted
Subject 1	0.0180	+	Female, Fed
Subject 2	0.0243	+	Male, Fasted
Subject 3	0.0250	+	Male, Fasted
Subject 3	+	not done	Male, Fed

[*] All subjects were non-smokers.

+ Identified.

TABLE 14

Ethylene Production by Particles from Rat Liver,

Rat Intestinal Mucosa and Penicillium digitatum

Run No.	Physical State	Ethylene (μl x 10^3/mg N/2 hr.)		
		Rat Liver	Rat Intestinal Mucosa	Penicillium digitatum
1	Untreated	25.1	–	–
1	Sonically treated	58.2*	16.17*	18.9
2	Untreated	5.0	35.87	12.5
3	Untreated	8.0	39.10	–
4	Sonically treated	9.5	35.80	13.7
5	Sonically treated	13.0	13.80	31.6
6	Sonically treated	–	12.50	28.6

* Mass spectrometric analysis confirmed the presence of

ethylene in these samples.

All fractions were sedimented at 12,728g for 15 minutes,

from the supernatant from centrifugation at 4,000g.

Reaction mixture as in Table 7.

clear-cut evidence for the production of ethylene by fractions from animal tissue.

Experiments were also conducted with particles separated from Penicillium digitatum, and the results are incorporated in Table 14. As with the animal tissue, this is the first report of ethylene production by fractions from homogenized fungal tissue. On a nitrogen basis, the production of ethylene (13-31 μl x 10^3/mg N) by particles of Penicillium digitatum is considerably lower than sonically treated tomato mitochondria (174 μl x 10^3/mg N, Table 11).

From these results, one is tempted to suggest that the production of ethylene may be a general phenomenon of all forms of life.

GENERAL DISCUSSION AND CONCLUSIONS

The original objective of the present investigation was to study the production of ethylene in cell-free systems of tomato. A pre-requisite to such a study was the development of a sensitive, quantitative method for determination of ethylene in the respiratory volatiles. Later the scope of the investigation was extended to ethylene production by particulate fractions of rat liver intestinal mucosa, and Penicillium digitatum. It was thought of interest also to analyse the respiratory gases of human subjects for ethylene.

A highly sensitive gas chromatographic unit with a hydrogen flame ionization detector was constructed. The structural and operational features of this instrument were critically examined. With our gas chromatographic unit we were able to detect 1 part of ethylene in 10^8 parts of air or respiratory gas. The response of our detector was linear, with a zero intercept, a significant improvement over the response of the detector constructed by Meigh (50). The best performance of the detector was found to depend not only on many structural features, but also on a critical combination of nitrogen and hydrogen in the carrier gas. With a mixture of nitrogen and hydrogen, each at a flow rate of 15 ml./2.6 seconds, we have achieved maximum column efficiency, high sensitivity, and overall electrical stability of the gas chromatographic unit.

A major difficulty in the study of ethylene production by sub-cellular fractions is the low rate of its emanation. This, of course, limits the availability of gas samples for quantitative analysis. It is obvious, therefore, that the collection efficiency should be very high, as well as reproducible; and the analytical procedures involving concentration and transfer of samples, etc., should be quantitative. Our method of collection, using a modified absorption tube, combined concentration of the gas and 100% collection efficiency, even at low concentrations of ethylene. In addition, in our procedure of regeneration of ethylene from the ethylene:mercury complex, we have combined simplicity and reproducibility. In fact, the entire procedure for collection of ethylene and subsequent gas chromatographic analysis had the desirable features of simplicity, high sensitivity, and quantitative reproducibility. Similar considerations apply to our procedure for collection and concentration of ethylene for mass spectrometric analysis.

We also have surmounted the difficulty (39) of positive identification of the gas as ethylene. In the first place, our method of ethylene collection in mercuric perchlorate was specific for olefins (63). Also, the retention time on the gas chromatographic column corresponded to that of ethylene. Added confirmation was obtained by co-chromatography with authentic ethylene and by disappearance

of the ethylene peak when the gas was passed through brominated activated carbon (47,48). Moreover, negative results for ethylene in mercuric perchlorate solutions aerated at the rate of 25 ml./minute for 4-16 hours in our collection assembly eliminated all possibility of contamination. These results together with corroborating evidence from mass spectrometric analysis confirmed that we were, in fact, dealing with ethylene.

Our first approach to the study of the production of ethylene was to investigate the general biochemical properties of the particulate fractions active in ethylene production. While there was considerable variation in these fractions, it was found that they were active in carbon dioxide production and phosphorylation, as well as in ethylene evolution, although no quantitative relationships among these activities could be expressed. Throughout this work we have used ethylene production rather than any other biochemical property as the main criterion in evaluation of the fractions and techniques used.

In Section IV, we have pointed out the consequences (with respect to ethylene production) of certain steps in the preparative procedure, particularly the particle washing and suspending techniques. As evident from yield and nitrogen analysis, the preparative procedure was not strictly quantitative. Moreover, the studies clearly questioned the use of washing procedures and all evidence indicated that

mitochondrial fragments may be present in many sub-cellular fractions. The most rewarding discovery from this phase of the work was that the ethylene-producing system was in some way related to the structural integrity of the mitochondrial particles. All results stressed the need for special purification techniques to enable one to relate the biochemical properties to the size and morphology of the sub-cellular particles.

Further experiments on preparative procedures clearly pointed out that ethylene production was related to the structural integrity of the particulate fractions. By eliminating washing of the particles and by carefully suspending the particles without mechanical assistance we were able to prepare "intact" mitochondria, which showed a lag period in carbon dioxide production (typical of intact mitochondria (72)) and no ethylene production. However, when the particles were allowed to age in vitro or deliberately disintegrated, the ethylene-producing system was activated. In this respect sonic treatment proved very effective.

By using quantitative comparisons it was possible to locate a site of the ethylene-producing system as the mitochondria and show that ethylene production was related to the structural integrity of these particles. This discovery offers an explanation of the existing controversy over the ability of mitochondria to produce ethylene.

Various procedures for initial grinding of the tissue and for washing and resuspension of sub-cellular fractions, as well as different centrifugal forces for isolation of the particles (and their fragments), would result in fractions of widely varying activity. Discarding of washings containing fragments of mitochondria could, for instance, result in absence of ethylene-producing activity. However, it is apparent that discrepancies in results may well arise from variations in the use of conventional techniques for preparation of cell fractions and for collection of ethylene. It must be emphasized that other investigators (36,37,39) have isolated and tested the particulate fractions, under conditions very different from ours. Full details of the preparative and analytical techniques and the biochemical properties of the particulate fractions were not reported by the other investigators, and hence it is not possible to draw detailed comparisons.

An added confirmation of our discovery that ethylene production by mitochondria was related to their structural integrity comes from our studies on the effects of calcium ions, cholic acid, and phospholipase A. All these reagents are well known to affect the permeability or structural integrity of mitochondria. All these reagents also bring about activation of ethylene production, although, under the conditions used, sonic treatment proved superior in this respect.

Our finding that ethylene production in vitro is associated with disintegration of mitochondria raises interesting questions with respect to ethylene production in vivo. It has been suggested (71) that mitochondria disintegrate as a fruit ripens. It is during ripening that ethylene evolution occurs. In this context, it would be of real interest to learn whether the production of ethylene is related to disintegration of mitochondria in vivo. Studies on the relation of ethylene production to the broader aspects of structure and function of mitochondria would also be of interest.

A prerequisite for any such study is a highly active cell-free system. Our attempts to prepare a particularly active system by isolation of a "combined mitochondria-microsomes" fraction did not succeed. On the contrary, we observed a definite inhibition of ethylene production. While many explanations on the basis of possible uncoupling of oxidative phosphorylation (24,25) or presence of natural inhibitors etc., can be advanced, further experiments need to be conducted before any valid conclusions can be reached. It is conceivable that, rather than a simple inhibition of the ethylene-producing system, the combination of the two fractions may provide an alternate, preferred pathway for utilization of ethylene.

Burg (17) has recently shown that, without exception, all fruits give off ethylene. While the production of ethylene

from microorganisms has been recognized for some time, it is only very recently that Kakanov (77) has provided evidence that normal rats and swine ascarids exhale ethylene. The formation of sub-cutaneous abcesses or sub-cutaneous transplantation of tumours resulted in an increased ethylene content of the exhaled gases of rat. These findings raise a pertinent question: How universal is the production of ethylene? Our analyses of the ethylene content of respired gases of human subjects, and the demonstration of ethylene production by sub-cellular particles from both _Penicillium digitatum_ and rat liver and intestinal mucosa suggest that the production of ethylene may be a general phenomenon.

Ethylene has been associated with many physiological changes leading to maturation and ripening in fruits (3 to 9), it is an industrial air pollutant (78), an anaesthetic agent, and has been claimed to be mutagenic and carcinogenic (77). Calvin (80) believes that ethylene was the first organic molecule synthesized in the evolutionary processes. Is ethylene a vestige of the evolutionary process or a vital physiological constituent common to all forms of life?

Until now, the relationship of ethylene to fruit ripening has been the main theme of study. Our findings open many new avenues for future studies on the biogenesis of ethylene and its physiological role in life processes.

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